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(54) Title: COMPOSITIONS AND METHODS FOR REGULATING ABCISIC ACID-INDUCED CLOSURE OF PLANT STOMATA

(57) Abstract: A novel gene, *AAPK*, is disclosed. Loss of function of the protein encoded by *AAPK* is associated with reduced sensitivity to abscisic acid-induced stomatal closure in plants. Also disclosed are transgenic plants and mutants having altered sensitivity to abscisic acid-mediated transpiration and other desirable agronomic features. The regulation of transpiration provided by the present invention is different from that of previously described mechanisms to control transpiration in plants.

Applicants : Josette Masle et al.
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COMPOSITIONS AND METHODS FOR REGULATING ABSCISIC ACID-INDUCED CLOSURE OF PLANT STOMATA

Pursuant to 35 U.S.C. §202(c), it is acknowledged that the U.S. Government has certain rights in the invention described herein, which was made in part with funds from the National Science Foundation, Grant Nos. MCB-9316319 and MCB-9874438.

5 This application claims priority to U.S. Provisional Application Nos. 60/142,039, filed July 1, 1999, 60/176,245, filed January 14, 2000 and 60/192,499, filed March 28, 2000, the entireties of which are incorporated by reference herein.

FIELD OF THE INVENTION

10 The present invention relates to the field of molecular biology of plants. More specifically, it relates to the regulation of gas exchange and transpirational water loss in plants possessing stomata.

BACKGROUND OF THE INVENTION

15 Various scientific and scholarly articles are referred to throughout the specification. These articles are incorporated by reference herein to describe the state of the art to which this invention pertains.

20 In terrestrial plants, water is transported, from the roots to the leaves, down a water potential gradient from the soil to the air. Transpiration, or loss of water from the leaves, helps create lowered osmotic potential in the leaves, effectively drawing water from the xylem to the mesophyll cells into the air spaces in the leaves. Estimates are that 90% or more of the water taken up by plants is lost to the air via transpiration.

25 Transpirational loss of water by evaporation occurs mainly through the pores, called stomata, primarily located in the lower epidermis of the leaves. Each stoma is surrounded by two guard cells, which control the opening and closure of the stomata by their relative turgor pressure. The cell wall properties of guard cells allow them to deform such that when the guard cells develop turgor pressure, the stoma is opened, but when the guard cells lose turgor, the stoma closes.

30 The rate of evaporation of water from the air spaces of the leaf to the outside air depends on the water potential gradient between the leaf and the outside air. Environmental factors which directly influence the aperture of the plant's stomata affect its transpiration rate. Such factors include light conditions, relative humidity of the air, temperature, water status of the plant, CO₂ concentration, relative concentration of certain ions, and concentration of abscisic acid (ABA).

Abscisic acid is a multifunctional phytohormone involved in a variety of

important protective functions including bud dormancy, seed dormancy and/or maturation, abscission of leaves and fruits, and response to a wide variety of biological stressors (e.g. cold, heat, salinity, and drought). It is also responsible for regulating stomatal closure by a mechanism independent of CO₂ concentration.

5 ABA is synthesized rapidly in response to water stress in plants, and is stored in the guard cells. During drought, ABA alteration of guard cell ion transport promotes stomatal closure and also prevents stomatal opening, thus reducing transpirational water loss. At the biochemical level, it is believed that the hormone sets off a variety of biological messages that require or include a protein phosphorylation 10 cascade. One member of this cascade was identified in guard cells of *Vicia faba* as an ABA-activated, calcium-independent protein kinase. (Li & Assmann, *Plant Cell* 8: 2359-2368, 1996; Mori & Muto, *Plant Physiol.* 113: 833-839, 1997). The kinase was identified by SDS polyacrylamide gel electrophoresis as a 48 kDa protein, but was not further isolated or characterized. It exhibited ABA-activated 15 autophosphorylation and kinase activity.

Stomata simultaneously regulate both the transpiration of water and the exchange of gases for photosynthesis. Open stomata allow for maximum gas exchange rate so that photosynthetic reactions may proceed more quickly, however under these conditions, water loss will be maximal. On the other hand, closed stomata minimize 20 transpirational water loss but also substantially reduce photosynthetic reaction rates. Paradoxically, the plant undergoes a continual trade-off between maximizing CO₂ uptake for carbon fixation, and minimizing desiccating water loss. Thus, the ability to control stomatal opening and closure could be of tremendous agronomic significance.

Several studies in the literature provide examples of the benefits of 25 selecting for increased stomatal conductance under certain conditions. One system that has been studied extensively comprises eight lines of Pima cotton (*Gossypium barbadense*) obtained over 40 years of selection and showing a 3-fold range in yield. These and additional studies have confirmed the association of higher conductance with 30 higher yield, and its genetic basis, in both Pima and Upland (*Gossypium hirsutum*) cotton. A similar correlation of increased yield, increased stomatal conductance, and decreased canopy temperatures has also been observed in a historical series of bread wheat cultivars. Taken collectively, this body of research suggests that selection or 35 genetic engineering of plants to achieve increased stomatal conductance may be of widespread utility for crop plants grown under irrigation under supra-optimal temperatures.

The plant hormone abscisic acid (ABA) causes stomatal closure during periods of reduced water availability by reducing the ion and water content of the pair of guard cells that flanks each stoma. However, even when plants are well-watered, ABA still limits stomatal aperture, as shown by the fact that mutants of tomato and

Arabidopsis that are deficient in either ABA-production or ABA-sensing have larger stomatal apertures than wild-type plants, even when water is plentiful. In other words, the ABA response is protective; always somewhat limiting to water loss, but thus unavoidably, also limiting to CO₂ uptake. This ABA-mediated limitation of water loss 5 is of no benefit however, to the grower who irrigates crops so that they are always well-watered. For those crops, such as many of the agricultural crops that are grown in arid or semi-arid regions, if this endogenous ABA-response of the stomata were “turned off”, crop yield could be increased, or the time for the plant to reach maturity decreased by removing the limits on increased CO₂ uptake and fixation.

10 Many crops, for example feed corn and wheat, are dried in the field before harvest. Other crops, such as tobacco and dried fruits such as raisins and prunes, are dried immediately post-harvest. It would be advantageous to growers to be able to accelerate or control the rate of crop drying.

15 For example, at the end of the growing season, it might be advantageous to dry the plants as quickly as possible, to minimize exposure to adverse weather conditions. However, water stress inevitably triggers ABA production/redistribution in the plant, leading to stomatal closure, which slows the rate of water loss, thus slowing the rate of crop drying. Therefore, it would be 20 advantageous to growers if this ABA-triggered stomatal closure response could be prevented or controlled.

25 In other cases, post-harvest, for many fruits, vegetables, and for cut-flowers, it is advantageous for the produce to dry out as slowly as possible, to retain freshness during transport, distribution, and purchase of the product. In these situations, it would be advantageous if the ABA-induced stomatal closure response could be enhanced. This could significantly extend the shelf life of the product.

The theoretical solution to the problems posed above is for growers to be able to precisely control the plant's transpiration via ABA-responsiveness of the guard cells/stomata. Ideally this control should be:

30 1. specific to the guard cells. It should not disrupt the many other effects of ABA on plant growth and development.

35 2. specific to ABA. There are many other stimuli that guard cells respond to in the control of stomatal aperture, for example, light and decreased intracellular concentrations of CO₂ drive stomatal opening, and conversely, darkness and high CO₂ concentrations drive stomatal closure. For crops under irrigation for example, the grower would still want the stomata to close in response to darkness, because in darkness there is no photosynthesis anyway, and open stomata during the night would simply waste irrigation water and thus money.

3. inducible, titratable, and reversible. To be of greatest utility, the grower would want to be able to control “when” and “how much” the guard cells

respond to ABA. Ideally, the grower would be able to open and close the stomata depending on the prevailing environmental conditions and desired results for his crop.

In present practice, growers have only limited control of rates of water loss from plants, mainly by controlling irrigation regimens in the field, and controlling environmental conditions during shipping, handling and storage of the product. By capitalizing on the present invention, growers could choose when plants would retain their maximum hydrated status (e.g. during times of water restriction, or for shipping of fresh produce), and when plants could be induced to dry out more quickly (e.g. as required for crops that are dried in the field before harvest).

Mutants have been identified in *Arabidopsis*, which display reduced rates of ABA production (*aba* mutants; Koornneef et al. 1982, *Theor. Appl. Genet.* 61:385-393) or ABA sensing (*abi* mutants; Koornneef et al. 1984 *Physiol. Plant.* 61:377-383). While these plants exhibit increased rates of water loss, the mutations are pleiotropic and this is a disadvantage. For example, the *aba* and *abi* mutants have reduced seed dormancy, and so the viability of the seed is likely to be reduced, a severe problem limiting any commercial application.

The isolation of novel mutants and genes that encode altered ABA-mediation of transpirational water loss will broaden the range of options for growers. It would be particularly advantageous to isolate mutants or genes involved in altered ABA-mediation of transpiration without spontaneously occurring abnormal responses to other roles of ABA or abnormal responses to factors such as light levels and concentrations of CO₂. Novel regulatory mutants are likely to have distinct induction of unique subsets of genes. The isolation of mutants will yield the critical gene(s) involved with altered ABA-mediation of transpiration, which can be used to transgenically transfer the novel trait to other species.

SUMMARY OF THE INVENTION

Provided in the present invention is a novel nucleic acid molecule (referred herein as AAPK), which is associated with regulation of transpiration by the hormone abscisic acid (ABA) in plants. The invention further provides transgenic plants and mutants having modified ABA-mediated stomatal closure. In these plants, ABA-mediated stomatal closure is modified in a manner that is independent of CO₂ and light-mediated responses of transpiration, as measured by changes in stomatal aperture.

According to one aspect of the present invention, a nucleic acid molecule encoding an ABA-activated protein kinase, AAPK, is provided. An exemplary AAPK-encoding nucleic acid molecule of the invention is that of *Vicia faba*, a food crop of major importance in the Middle East. Also exemplified are homologs of the gene in *Arabidopsis thaliana*. The invention further provides

homologs of the exemplified *AAPK*, having a level of nucleotide sequence or amino acid sequence identity with the exemplified *AAPK* nucleic acids or encoded *AAPK* proteins, specifically at certain regions of the coding sequence, that clearly distinguish the homologs as *AAPK* homologs, as opposed to other kinases. Preferably, these 5 homologs comprise nucleotide or amino acid sequences at least 60%, preferably 67%, more preferably 70% and even more preferably 80% identical to the *Vicia faba* and *Arabidopsis* *AAPK* nucleic acid and *AAPK* amino acid sequences set forth herein..

Also provided in accordance with the present invention is a disrupted gene product of the *AAPK* gene. In a preferred embodiment, the disrupted gene 10 product comprises lost or reduced activity of the *AAPK* protein. Reduction in amount or activity of *AAPK* in plants results in decreased sensitivity of the plants to ABA-induced stomatal closure, but does not affect the plants's sensitivity to dark- or CO₂-induced stomatal closure.

According to another aspect of the invention, an oligonucleotide 15 molecule of at least 15 nucleotides in length, preferably at least 20 nucleotides in length, and most preferably at least 30 nucleotides in length, that is identical in sequence to a portion of an *AAPK* nucleic acid, is provided. In a preferred embodiment, the invention provides a nucleic acid molecule of at least 15, preferably 20, and most 20 preferably 30 or more nucleotides in length, that is identical to or complementary to a consecutive 15, 20 or 30 nucleotide portion, respectively, of the sequence set out in one of SEQ ID NOS:1, 3 or 6.

According to other aspects of the invention, an isolated polypeptide produced by expression of a nucleic acid molecule of the invention is provided. Also featured are antibodies immunologically specific for such a polypeptide.

According to another aspect of the invention, a vector for transforming 25 a plant cell, comprising a nucleic acid molecule of the invention, is provided. Also featured are plant cells transformed with the vector, and intact fertile plants regenerated from the plant cells. It will be appreciated by persons of skill in the art, that various portions of such genetically altered plants are also encompassed by the present invention. These include, but are not limited to, roots, modified roots (e.g., tubers), 30 stems, leaves, flowers, fruits and seeds, and components thereof, e.g., extracts or oils.

According to another aspect of the invention, a genetically altered plant 35 is provided, which possesses decreased sensitivity to ABA-induced stomatal closure as compared with an equivalent but unaltered plant. These genetically altered plants contain an *AAPK* that is largely nonfunctional or absent. In one embodiment, the plant is produced by subjecting a population of plants to mutagenesis and selecting a mutagenized plant wherein the *AAPK* is largely nonfunctional or absent. In a preferred embodiment, the plant is produced by transforming cells of the plant with a transgene that causes the plant's endogenous *AAPK* to become largely nonfunctional or absent,

and regenerating the plant from the transformed cell. In a particularly preferred embodiment, expression of the transgene is inducible.

According to another aspect of the invention, a genetically altered plant possessing increased sensitivity to ABA-induced stomatal closure as compared with an equivalent but unaltered plant, is provided. Plants of this type contain an AAPK that is increased in amount or activity as compared with the unaltered plant. In one embodiment, these plants are produced by subjecting a population of plants to mutagenesis and selecting a mutagenized plant wherein the AAPK is largely nonfunctional or absent. In a preferred embodiment, the plants are produced by transforming cells of the plant with a transgene that causes the plant's endogenous AAPK to become largely nonfunctional or absent, and regenerating the plant from the transformed cells. In a particularly preferred embodiment, expression of the transgene is inducible.

Another aspect of the invention features a method to increase transpiration in a plant. The method comprises reducing or preventing function of an AAPK in guard cells of the plant, thereby reducing sensitivity of the plant to ABA-induced stomatal closure, resulting in the increased transpiration. Conversely, a method is provided to decrease transpiration in a plant, comprising increasing function of AAPK in guard cells of the plant, thereby increasing sensitivity of the plant to ABA-induced stomatal closure, resulting in decreased transpiration.

Other features and advantages of the present invention will be better understood by reference to the drawings, detailed description and examples that follow.

25

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Alignment of the deduced AAPK amino acid sequence with those of homologous protein kinases. GenBank accession numbers for the nucleic acid molecules encoding the displayed amino acid sequences are: AAPK (AF186020), 30 *Arabidopsis* Atpk (L05562, S71172), tobacco WAPK (AF032465), soybean SPK-4 (L38855), rice REK (AB002109), ice plant MK9 (Z26846), and wheat PKABA1 (M94726). Sequence ID Numbers for the displayed sequences are as follows: AAPK is SEQ ID NO:2 (encoded by SEQ ID NO:1); Atpk is SEQ ID NO:4 (encoded by SEQ ID NO:3); WAPK is SEQ ID NO: 11; SPK-4 is SEQ ID NO:12; 35 REK is SEQ ID NO:13; MK9 is SEQ ID NO:14; PKABA1 is SEQ ID NO: 10 (encoded by SEQ ID NO:9). Amino acids are highlighted when there are at least four identical residues among the seven sequences. Conserved subdomains of the protein kinase family are indicated by roman numerals. Peptide sequences obtained by tandem mass spectrometry are marked by lines. Peptide regions used for designing degenerate

PCR primers are indicated by arrows. Sequences were aligned by the Clustal method in MegAlign (DNASTAR, Madison, WI). Numbers indicate amino acid positions.

5 **Figure 2.** Alignment of the deduced amino acid sequence of AAPK from *Vicia faba* (SEQ ID NO:2) with the amino acid sequence of a homologous protein kinase from *Arabidopsis thaliana* (SEQ ID NO:5). Query sequence = AAPK (GI 6739629), Length = 349 amino acids. Subject sequence = *A. thaliana* protein kinase, Length = 357 amino acids [GenBank Accession Number CAA19877 (GI 3297819)]. Comparison was done using the Blast 2 alignment program at NCBI, with following default parameters: Matrix: BLOSUM62, Gap Penalties: Existence: 11, Extension: 1. Identities = 270/348 (77%), Positives = 311/348 (88%), Gaps = 1/348 (0%). The sequence shown between the Query and the Subject sequences shows the consensus sequence. A letter indicates identity, a '+' indicates a similarity, while a blank space indicates the two sequences are different at that residue.

10

15 **Figure 3.** Alignment of the deduced amino acid sequence of AAPK from *Vicia faba* (SEQ ID NO:2) with the deduced amino acid sequence from a gene encoding a homologous protein kinase from *Arabidopsis thaliana* (SEQ ID NO:7). Query sequence = AAPK (GI 6739629), Length = 349 amino acids. Subject sequence = deduced amino acid sequence from *A. thaliana* L05561 clone, Length = 362 amino acids [GenBank Accession Number L05561 (GI 166817)]. Comparison 20 was done using the Blast 2 alignment program at NCBI, with following default parameters: Matrix: BLOSUM62, Gap Penalties: Existence: 11, Extension: 1. Identities = 273/353 (77%), Positives = 318/353 (89%), Gaps = 4/353 (1%). The sequence shown between the Query and the Subject sequences shows the consensus sequence. A letter indicates identity, a '+' indicates a similarity, while a blank space indicates the two sequences are different at that residue.

25

30 **Figure 4.** Alignment of the deduced amino acid sequence of AAPK from *Vicia faba* (SEQ ID NO:2) with the amino acid sequence of a homolog, Protein Kinase SPK-2, from *Arabidopsis thaliana* (SEQ ID NO:8). Query sequence = AAPK (GI 6739629), Length = 349 amino acids. Subject sequence = amino acid sequence from *A. thaliana*, Length = 362 amino acids [GenBank Accession Number S56718 (GI 1362002)]. Comparison was done using the Blast 2 alignment program at NCBI, with following default parameters: Matrix: BLOSUM62, Gap Penalties: Existence: 11, Extension: 1. Identities = 273/353 (77%), Positives = 318/353 (89%), Gaps = 4/353 (1%). The sequence shown between the Query and the Subject 35 sequences shows the consensus sequence. A letter indicates identity, a '+' indicates a similarity, while a blank space indicates the two sequences are different at that residue.

DETAILED DESCRIPTION OF THE INVENTION**I. Definitions**

Various terms relating to the biological molecules of the present invention are used hereinabove and also throughout the specifications and claims.

5 With respect to the genotypes of the invention, the term "AAPK" is used to designate the naturally-occurring or wild-type genotype. This genotype has the phenotype of naturally-occurring sensitivity to the effects of ABA. Where used hereinabove and throughout the specifications and claims, the term "AAPK" refers to the protein product of the *AAPK* gene.

10 In reference to the mutants of the invention, the term "null mutant" or "loss-of-function mutant" is used to designate an organism or genomic DNA sequence with a mutation that causes the product of the gene of interest to be non-functional or largely absent. Such mutations may occur in the coding and/or regulatory regions of the gene of interest, and may be changes of individual residues, or insertions or 15 deletions of regions of nucleic acids. These mutations may also occur in the coding and/or regulatory regions of other genes which may regulate or control the gene of interest and/or its encoded gene product so as to cause said gene product to be non-functional or largely absent.

20 With reference to certain of the DNA constructs of the invention, the terms "pGFP", "pAAPK-GFP" and "pAAPK(K43A)-GFP" refer to constructs made from the green fluorescent protein (GFP) expression vector, pGFP, which allows cells transformed with the pGFP to express the readily-detected green fluorescent protein. Where used herein, "pAAPK-GFP" refers to a pGFP expression vector with the sequence encoding AAPK inserted upstream of and in-frame with the sequence 25 encoding the GFP, such that both proteins can be expressed in cells transformed with this construct. Where used herein, "pAAPK(K43A)-GFP" refers to a pGFP expression vector with the sequence encoding an AAPK, modified such that the lysine residue at position 43 in the ATP binding site is changed to an alanine, inserted upstream of and in-frame with the sequence encoding the GFP, such that both proteins 30 can be expressed in cells transformed with this construct.

With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to genomic DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally-occurring genome of the 35 organism from which it was derived. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a prokaryote or eukaryote. An "isolated nucleic acid molecule" may also comprise a cDNA molecule or a synthetic DNA molecule.

With respect to RNA molecules of the invention, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form.

Nucleic acid sequences and amino acid sequences can be compared using computer programs that align the similar sequences of the nucleic or amino acids thus define the differences. In preferred methodologies, the BLAST programs (NCBI) and parameters used therein are employed to align nucleotide and amino acid sequences. However, equivalent alignments and similarity/identity assessments can be obtained through the use of any standard alignment software. For instance, the DNAsstar system (Madison, WI) may be used to align sequence fragments of genomic or other DNA sequences. Alternatively, GCG Wisconsin Package version 9.1, available from the Genetics Computer Group in Madison, Wisconsin, and the default parameters used (gap creation penalty=12, gap extension penalty=4) by that program may also be used to compare sequence identity and similarity.

The term "substantially the same" refers to nucleic acid or amino acid sequences having sequence variation that do not materially affect the nature of the protein (i.e. the structure, stability characteristics and/or biological activity of the protein). With particular reference to nucleic acid sequences, the term "substantially the same" is intended to refer to the coding region and to conserved sequences governing expression, and refers primarily to degenerate codons encoding the same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially the same" refers generally to conservative substitutions and/or variations in regions of the polypeptide not involved in determination of structure or function.

The terms "percent identical" and "percent similar" are also used herein in comparisons among amino acid and nucleic acid sequences. When referring to amino acid sequences, "percent identical" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical amino acids in the compared amino acid sequence by a sequence analysis program. "Percent similar" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical or conserved amino acids. Conserved amino acids are those which differ in structure but are similar in physical properties such that the exchange of one for another would not appreciably change the tertiary structure of the resulting protein. Conservative substitutions are defined by Taylor (1986, *J. Theor. Biol.* 119:205). When referring to nucleic acid molecules, "percent identical" refers to the percent of the nucleotides of the subject nucleic acid sequence that have been matched

to identical nucleotides by a sequence analysis program.

With respect to protein, the term "isolated protein" or "isolated and purified protein" is sometimes used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention.

5 Alternatively, this term may refer to a protein which has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form. In this regard, "isolated" or "isolated and purified" also refers to its separation or removal from a chromatography column matrix or a gel, such as a polyacrylamide gel. That is, a polypeptide that has been separated by chromatography

10 or polyacrylamide gel electrophoresis, but is not eluted from the matrix or gel, is not considered "isolated" or "isolated and purified".

With respect to antibodies of the invention, the terms "immunologically specific" or "specific" refer to antibodies that bind to one or more epitopes of a protein of interest, but which do not substantially recognize and bind other molecules in a

15 sample containing a mixed population of antigenic biological molecules.

With respect to single-stranded nucleic acid molecules, the term "specifically hybridizing" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed

20 "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

25 A "coding sequence" or "coding region" refers to a nucleic acid molecule having sequence information necessary to produce a gene product, when the sequence is expressed.

The term "operably linked" or "operably inserted" means that the regulatory sequences necessary for expression of the coding sequence are placed in a nucleic acid molecule in the appropriate positions relative to the coding sequence so as to enable expression of the coding sequence. This same definition is sometimes applied to the arrangement of other transcription control elements (e.g. enhancers) in an expression vector.

30 Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

35 The terms "promoter", "promoter region" or "promoter sequence" refer generally to transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns. Typically, a promoter is a DNA regulatory region capable of binding RNA polymerase

in a cell and initiating transcription of a downstream (3' direction) coding sequence. The typical 5' promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background.

5 Within the promoter sequence is a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

10 A "vector" is a replicon, such as plasmid, phage, cosmid, or virus to which another nucleic acid segment may be operably inserted so as to bring about the replication or expression of the segment.

15 The term "nucleic acid construct" or "DNA construct" is sometimes used to refer to a coding sequence or sequences operably linked to appropriate regulatory sequences and inserted into a vector for transforming a cell. This term may be used interchangeably with the term "transforming DNA". Such a nucleic acid construct may contain a coding sequence for a gene product of interest, along with a selectable marker gene and/or a reporter gene.

20 The term "selectable marker gene" refers to a gene encoding a product that, when expressed, confers a selectable phenotype such as antibiotic resistance on a transformed cell.

25 The term "reporter gene" refers to a gene that encodes a product which is easily detectable by standard methods, either directly or indirectly.

30 A "heterologous" region of a nucleic acid construct is an identifiable segment (or segments) of the nucleic acid molecule within a larger molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, a heterologous region is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein. The term "DNA construct", as defined above, is also used to refer to a heterologous region, particularly one constructed for use in transformation of a cell.

35 A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has

become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a 5 single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

II. Description

In accordance with the present invention, an isolated nucleic acid 10 molecule is provided that encodes a novel regulator of ABA-mediated stomata aperture control. This nucleic acid molecule is referred to herein as AAPK ("ABA-activated protein kinase"). Its manner of regulating ABA-mediated stomata aperture control is novel and interesting. When the functional product of the gene is eliminated or specifically altered in a plant, the plant exhibits decreased sensitivity to ABA-induced stomatal closure, but without changing the plant's responses to stomatal closure induced by darkness or CO₂. In addition, the loss of function of AAPK had 15 no effect on ABA-mediated inhibition of stomatal opening. While not intending to limit the present invention by describing one possible mechanism of action of AAPK, it may be that AAPK functions as a negative regulator of ABA-mediated stomatal aperture control, and resultant transpiration and gas exchange.

AAPK was selected as an important target for cloning firstly because 20 its ABA-activated phosphorylation activity is specific to guard cells. Secondly, AAPK was selected because it was not activated by other stoma-closing factors such as CO₂ or darkness, making it a potential guard cell-specific, ABA response regulator of 25 stomatal closure. Furthermore, since maintaining control of transpirational water loss and gas exchange is of a vital and fundamental nature to plants, AAPK is likely to be a highly conserved function among all plant species.

The AAPK cDNA was isolated from a *Vicia faba* cDNA library by 30 using degenerate primers created based on peptide sequence data. The degenerate primers were used in reverse transcriptase – PCR to generate a 310 bp probe from guard cell RNA. The probe was then used to screen a *V. faba* guard cell cDNA library. Based on analysis of the probes, the AAPK gene product appears to be a 35 significant protein kinase in the guard cells, inasmuch as other protein kinases were not identified despite the fact that the probe was homologous to domains common to other protein kinase family members. Sequence analysis of this cDNA revealed the nucleic acid sequence of SEQ ID NO:1, and a predicted polypeptide sequence having SEQ ID NO:2. The deduced amino acid sequence was compared to PKABA1 (SEQ ID NO:10), the expressed product of an ABA-induced transcript from wheat (Anderberg & Walker-Simmons, Proc. Natl. Acad. Sci. USA 89: 10183-10187, 1992).

PKABA1 is a known protein kinase with conserved regions common to this family of kinases. Comparison with PKABA1 revealed that AAPK also possesses these conserved kinase domains.

Many diverse protein kinases are involved in cascading cellular signal transduction; however the kinase domain is highly conserved in all protein kinases. The AAPK protein sequence contains high similarity to a large number of protein kinases, as revealed, for example, by the alignment of plant protein kinases shown in Figure 1. The functional specialization that allows these kinases to operate in specific signal transduction pathways lies both in the kinase domain and non-kinase domains. The *Vicia faba* AAPK kinase protein (SEQ ID NO:2) displays significant similarity to PKABA1 (SEQ ID NO:10). While the similarity is highest in the putative kinase domains, there are several regions where the two proteins are less different from one another. PKABA is expressed from an ABA-induced transcript, but it has not been shown to possess the ABA-activated protein kinase activity of AAPK, suggesting that it plays a different role.

As described in Example 2, genomic screening of an *Arabidopsis* library and GenBank database screening using the SEQ ID NO:1 cDNA reveals that the *Vicia faba* AAPK is most similar to the proteins (SEQ ID NOS: 4, 5 and 7) encoded by the *Arabidopsis* genes having Genbank Accession Numbers L05561 and L05562, and *Arabidopsis* protein having Accession No. CAA19877, respectively, indicating that these genes and proteins are clear functional homologs of *Vicia faba* AAPK and its encoded protein.

An additional round of database screening was performed, using peptide segments of SEQ ID NO:2 that were homologous to SEQ ID NO: 4 (encoded by GenBank L05562, *Arabidopsis* Atpk) but different from wheat PKABA1 (SEQ ID NO:10). These peptides were: (1) PIMHDSDRYDF (SEQ ID NO:15), corresponding to residues 5-15 of SEQ ID NO:2 at the amino terminus; and (2) PADLVNENIMDNQFEEPDQ (SEQ ID NO:16), corresponding to residues 275-293 of SEQ ID NO:2 near the carboxyl terminus. Screening with either of these peptides corroborated the physical and database screening using the complete sequence, identifying each the aforementioned *Arabidopsis* proteins. This screening also revealed a fourth homolog, identified in the database as Protein Kinase SPK-2, Accession No. S56718 (SEQ ID NO:8). It is possible that this *Arabidopsis* protein is the same as the predicted protein from clone L05561.

Although the AAPK cDNA clone from *Vicia faba*, and homologs from *Arabidopsis* are described and exemplified herein, this invention is intended to encompass nucleic acid sequences and proteins from other plants that are sufficiently similar to be used instead of the *Vicia faba* or *Arabidopsis* AAPK nucleic acids and proteins for the purposes described below. These include, but are not limited to, allelic

variants and natural mutants of *AAPK*, which are likely to be found in different varieties of *Vicia faba*, as well as homologs of *AAPK* from different species of plants. Because such variants and homologs are expected to possess certain differences in nucleotide and amino acid sequence, this invention provides an isolated *AAPK* nucleic acid

5 molecule having at least about 50% (preferably 60%, more preferably 70% and even more preferably over 80%) sequence identity in the coding regions with the nucleotide sequence set forth as SEQ ID NOS: 1, 3, 5, 7 or 9 (and, most preferably, specifically comprising the coding regions of SEQ ID NOS: 1, 3, or 6. This invention also provides isolated polypeptide products of the open reading frames of *AAPK*, having at least

10 about 60% (preferably 70%, 75%, 80% or greater) sequence identity with the amino acid sequences of SEQ ID NOS: 2, 4, 5, 7 or 9.. Because of the natural sequence variation likely to exist among *AAPK* genes, one skilled in the art would expect to find up to about 30-40% nucleotide sequence variation, while still maintaining the unique properties of the *AAPK* nucleic acid molecules and encoded polypeptides of the

15 present invention. Such an expectation is due in part to the degeneracy of the genetic code, as well as to the known evolutionary success of conservative amino acid sequence variations, which do not appreciably alter the nature of the encoded protein. Accordingly, such variants and homologs are considered substantially the same as one another and are included within the scope of the present invention. Within the

20 parameters of sequence identity and similarity set forth above, *AAPKs* from any plant species are considered part of the present invention. Such plant species include dicotyledenous and monocotyledenous flowering plants, as well as any other plant that possesses stomata. Of particular importance to the invention are *AAPKs* from agronomically or horticulturally important plant species, including maize, wheat, rye,

25 oats, barley, rice, sorghum, soy and other beans, alfalfa, sunflower, canola, lawn and turfgrasses, tobacco, aster, zinnia, chrysanthemum, beet, carrot, cruciferous vegetables, cucumber, grape, pea, potato, rutabaga, tomato, tomatillo and turnip, to name a few.

AAPK nucleic acid molecules of the invention include DNA, RNA, and fragments thereof which may be single- or double-stranded. Thus, this invention

30 provides oligonucleotides (sense or antisense strands of DNA or RNA) having sequences capable of hybridizing with at least one sequence of a nucleic acid molecule encoding the protein of the present invention. Such oligonucleotides are useful as probes for detecting *AAPK* genes or transcripts.

In addition to encompassing natural mutants of *AAPK*, the present

35 invention is drawn to artificially created mutants, produced by *in vitro* mutagenesis or isolated from mutagenized plants, as described in greater detail below. These mutant *AAPK* nucleic acids and their encoded proteins are integral to practicing the methods of the invention, which involve regulating ABA-mediated stomatal closure in plants. The present invention further encompasses genetically modified plants having altered

transpiration and gas exchange characteristics due to the down-regulation or up-regulation of AAPK in those plants.

The following sections set forth the general procedures involved in practicing the present invention in all of its aspects as summarized above. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general cloning procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (hereinafter "Sambrook et al.") or Ausubel et al. (eds) Current Protocols in Molecular Biology, John Wiley & Sons (2000) (hereinafter "Ausubel et al.") are used.

A. Preparation of AAPK nucleic acids, proteins, antibodies, AAPK mutants and transgenic plants

15 **Preparation of AAPK Nucleic Acid Molecules.** AAPK nucleic acid molecules of the invention may be prepared by two general methods: (1) they may be synthesized from appropriate nucleotide triphosphates, or (2) they may be isolated from biological sources. Both methods utilize protocols well known in the art.

20 The availability of nucleotide sequence information, such SEQ ID NOS: 1, 3 and 6, enables preparation of an isolated nucleic acid molecule of the invention by polynucleotide synthesis. Synthetic oligonucleotides may be prepared by the phosphoramidite method employed in the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant construct may be purified according to methods known in the art, such as high performance liquid chromatography (HPLC).

25 Long, double-stranded polynucleotides, such as a DNA molecule of the present invention, must be synthesized in stages, due to the size limitations inherent in current oligonucleotide synthetic methods. Thus, for example, a long double-stranded molecule may be synthesized as several smaller segments of appropriate complementarity. Complementary segments thus produced may be annealed such that 30 each segment possesses appropriate cohesive termini for attachment of an adjacent segment. Adjacent segments may be ligated by annealing cohesive termini in the presence of DNA ligase to construct an entire long double-stranded molecule. A synthetic DNA molecule so constructed may then be cloned and amplified in an appropriate vector.

35 Modified (i.e., "mutant") nucleic acid molecules of the invention also may be synthesized as described above. In this embodiment, the desired alteration is simply programmed into the synthetic scheme. In another embodiment, an unaltered synthetic nucleic acid molecule is manufactured, and subsequently altered by site-directed mutagenesis.

Nucleic acid molecules encoding the AAPK protein may be isolated from *V. faba*, *Arabidopsis* or any other plant of interest using methods well known in the art. It will be appreciated that such methods may be used to screen libraries of mutant plants as well as wild-type plants. In order to isolate AAPK-encoding nucleic acids from plants other than *V. faba*, or *Arabidopsis*, oligonucleotides designed to match the nucleic acids encoding the *V. faba* or *Arabidopsis* AAPK protein may be used with cDNA or genomic libraries from the desired species. If the *AAPK* gene from a species is desired, the genomic library is screened. Alternately, if the protein coding sequence is of particular interest, the cDNA library is screened. In positions of degeneracy, where more than one nucleic acid residue could be used to encode the appropriate amino acid residue, all the appropriate nucleic acids residues may be incorporated to create a mixed oligonucleotide population, or a neutral base such as inosine may be used. Such degenerate libraries also may be customized for the codon preference of the plant species to be screened. The strategy of oligonucleotide design is well known in the art (see Ausbel et al., Sambrook et al.).

In another embodiment, known *AAPK* sequences may be used in "data mining" to screen databases for homologous sequences, as is well known in the art and exemplified herein.

Alternatively, PCR (polymerase chain reaction) primers may be designed by the above method to encode a portion a known AAPK protein, and these primers used to amplify nucleic acids from isolated cDNA or genomic DNA. In a preferred embodiment, the oligonucleotides used to isolate *AAPK* nucleic acids are designed to encode sequences conserved among AAPKs, but not between AAPK and other kinases (e.g., the PKABA1 protein kinase family), as described above.

In accordance with the present invention, nucleic acids having the appropriate sequence homology with a known *AAPK* nucleic acid molecule may be identified by using hybridization and washing conditions of appropriate stringency. For example, hybridizations may be performed, according to the method of Sambrook et al. (1989, *supra*), using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65° in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology (Sambrook et al.) is:

$$T_m = 81.5EC + 16.6\log [Na^+] + 0.41(\% G+C) - 0.63 (\% formamide) - 600/\#bp \text{ in duplex}$$

As an illustration of the above formula, using $[N^+] = [0.368]$ and 50% formamide, with
5 GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C. In a preferred embodiment, the hybridization is at 37°C and the final wash is at 42°C, in a more preferred embodiment the hybridization
10 is at 42°C and the final wash is at 50°C, and in a most preferred embodiment the hybridization is at 42°C and final wash is at 65°C, with the above hybridization and wash solutions. Conditions of high stringency include hybridization at 42°C in the above hybridization solution and a final wash at 65°C in 0.1X SSC and 0.1% SDS for 10 minutes.

15 Nucleic acids of the present invention may be maintained as DNA in any convenient cloning vector. In a preferred embodiment, clones are maintained in plasmid cloning/expression vector, such as pBluescript (Stratagene, La Jolla, CA), which is propagated in a suitable *E. coli* host cell.

20 **Preparation of polypeptides and antibodies.** AAPK polypeptides may be prepared in a variety of ways, according to known methods. The availability of nucleic acid molecules encoding the polypeptides enables synthesis of the proteins by known methods, or production of the proteins using *in vitro* expression methods known in the art. For example, a cDNA or gene may be cloned into an appropriate *in vitro* transcription vector, such a pSP64 or pSP65 for *in vitro* transcription, followed
25 by cell-free translation in a suitable cell-free translation system, such as wheat germ or rabbit reticulocytes. *In vitro* transcription and translation systems are commercially available, e.g., from Promega Biotech, Madison, Wisconsin or BRL, Rockville, Maryland. The pCITE *in vitro* translation system (Novagen) also may be utilized.

30 According to a preferred embodiment, larger quantities of the proteins may be produced by expression in a suitable prokaryotic or eukaryotic system. For example, part or all of a DNA molecule, such as the coding portion of SEQ ID NOS: 1, 3 or 6, or appropriate complementary sequences, may be inserted into a plasmid vector adapted for expression in a bacterial cell (such as *E. coli*) or a yeast cell (such as *Saccharomyces cerevisiae*), or into a baculovirus vector for expression in an insect cell. Such vectors comprise the regulatory elements necessary for expression of the DNA in the host cell, positioned in such a manner as to permit expression of the DNA in the host cell. Such regulatory elements required for expression include promoter sequences, transcription initiation sequences and, optionally, enhancer sequences.

35 The AAPK polypeptides produced by gene expression in a recombinant prokaryotic or eukaryotic system may be purified according to methods

known in the art. In a preferred embodiment, a commercially available expression/secretion system can be used, whereby the recombinant protein is expressed and thereafter secreted from the host cell, to be easily purified from the surrounding medium. If expression/secretion vectors are not used, an alternative 5 approach involves purifying the recombinant protein by affinity separation, such as by immunological interaction with antibodies that bind specifically to the recombinant protein. Such methods are commonly used by skilled practitioners.

10 AAPK proteins, prepared by the aforementioned methods, may be analyzed according to standard procedures. Methods for analyzing the functional activity of kinases are well known to persons skilled in the art. Alternatively, the function of the kinase in stomatal closure may be analyzed, as described in greater detail below and in Example 1.

15 The present invention also provides antibodies that are immunologically specific to the AAPK of the invention. Polyclonal antibodies may be prepared according to standard methods. In a preferred embodiment, monoclonal antibodies are prepared, which are specific to various epitopes of the protein. Monoclonal antibodies may be prepared according to general methods of Köhler and Milstein, following standard protocols. Polyclonal or monoclonal antibodies that are immunologically specific for the AAPK can be utilized for identifying and purifying 20 AAPK from *V. faba* and other species. For example, antibodies may be utilized for affinity separation of proteins for which they are specific or to quantify the protein. Antibodies may also be used to immunoprecipitate proteins from a sample containing a mixture of proteins and other biological molecules.

25 **Mutants and transgenic plants.** Example 1 describes a synthetic mutant, AAPK(K43A) in *Vicia faba*, which displays insensitivity to ABA-induce stomatal closure due to the loss of function of AAPK. Any plant may be transgenically engineered to display a similar phenotype. This approach is particularly appropriate to plants with high ploidy numbers, including but not limited to wheat.

30 These synthetic null mutant are created by expressing a mutant form of the AAPK protein to create a “dominant negative effect”. While not limiting the invention to any one mechanism, this mutant AAPK protein competes with wild-type AAPK protein for interacting proteins in the transgenic plant, or poisons an AAPK multimeric complex. By over-producing the mutant form of the protein, the signaling pathway used by the wild-type AAPK protein can be effectively blocked. Examples 35 of this type of “dominant negative” effect are well known for both insect and vertebrate systems (Radke et al, 1997, Genetics 145:163-171; Kolch et al., 1991, Nature 349:426-428). In a preferred embodiment, the mutant protein is produced by mutating the coding sequence of AAPK corresponding to residues in the active site. In a particularly preferred embodiment, the coding sequence corresponding to the lysine

residue at position 43 is mutated to code for a different, preferably non-similar, amino acid residue, for example, alanine.

A second kind of synthetic null mutant can be created by inhibiting the translation of the AAPK mRNA by "post-transcriptional gene silencing". The AAPK gene from the species targeted for down-regulation, or a fragment thereof, may be utilized to control the production of the encoded protein. Full-length antisense molecules or antisense oligonucleotides are used that are targeted to specific regions of the AAPK-encoded RNA that are critical for translation. The use of antisense molecules to decrease expression levels of a pre-determined gene is known in the art.

5 Antisense molecules may be provided *in situ* by transforming plant cells with a DNA construct which, upon transcription, produces the antisense RNA sequences. Such constructs can be designed to produce full-length or partial antisense sequences. This gene silencing effect can be enhanced by transgenically over-producing both sense and antisense RNA of the gene coding sequence so that a high amount of dsRNA is

10 produced (for example see Waterhouse et al., 1998, PNAS 95:13959-13964). In a preferred embodiment, part or all of the AAPK coding sequence antisense strand is expressed by a transgene. In a particularly preferred embodiment, hybridizing sense and antisense strands of part or all of the AAPK coding sequence are transgenically expressed.

15

20 A third type of synthetic null mutant can also be created by the technique of "co-suppression". Plant cells are transformed with a copy of the endogenous gene targeted for repression. In many cases, this results in the complete repression of the native gene as well as the transgene. In a preferred embodiment, the AAPK gene from the plant species of interest is isolated and used to transform cells of that same species.

25 Transgenic plants can also be created that have enhanced AAPK activity. This can be accomplished by transforming plant cells with a transgene that expresses part or all of the AAPK coding sequence, or a sequence that encodes the either the AAPK protein or a protein functionally similar to it. In a preferred embodiment, the complete AAPK coding sequence is transgenically over-expressed. In another embodiment, the coding sequence corresponding to the kinase domain of AAPK is over-expressed.

30 Transgenic plants with one of the transgenes mentioned above can be generated using standard plant transformation methods known to those skilled in the art. These include, but are not limited to, *Agrobacterium* vectors, polyethylene glycol treatment of protoplasts, biolistic DNA delivery, UV laser microbeam, gemini virus vectors, calcium phosphate treatment of protoplasts, electroporation of isolated protoplasts, agitation of cell suspensions in solution with microbeads coated with the transforming DNA, agitation of cell suspension in solution with silicon fibers coated

with transforming DNA, direct DNA uptake, liposome-mediated DNA uptake, and the like. Such methods have been published in the art. See, e.g., Methods for Plant Molecular Biology (Weissbach & Weissbach, eds., 1988); Methods in Plant Molecular Biology (Schuler & Zielinski, eds., 1989); Plant Molecular Biology Manual (Gelvin, Schilperoort, Verma, eds., 1993); and Methods in Plant Molecular Biology - A Laboratory Manual (Maliga, Klessig, Cashmore, Gruisse & Varner, eds., 1994).

5 The method of transformation depends upon the plant to be transformed. *Agrobacterium* vectors are often used to transform dicot species. *Agrobacterium* binary vectors include, but are not limited to, BIN19 (Bevan, 1984) 10 and derivatives thereof, the pBI vector series (Jefferson et al., 1987), and binary vectors pGA482 and pGA492 (An, 1986). For transformation of monocot species, biolistic bombardment with particles coated with transforming DNA and silicon fibers coated with transforming DNA are often useful for nuclear transformation.

15 DNA constructs for transforming a selected plant comprise a coding sequence of interest operably linked to appropriate 5' (e.g., promoters and translational regulatory sequences) and 3' regulatory sequences (e.g., terminators). In a preferred embodiment, the coding region is placed under a powerful constitutive promoter, such as the Cauliflower Mosaic Virus (CaMV) 35S promoter or the figwort mosaic virus 20 35S promoter. Other constitutive promoters contemplated for use in the present invention include, but are not limited to: T-DNA mannopine synthetase, nopaline synthase (NOS) and octopine synthase (OCS) promoters.

25 Transgenic plants expressing a sense or antisense AAPK coding sequence under an inducible promoter are also contemplated to be within the scope of the present invention. Inducible plant promoters include the tetracycline repressor/operator controlled promoter, the heat shock gene promoters, stress (e.g., 30 wounding)-induced promoters, defense responsive gene promoters (e.g. phenylalanine ammonia lyase genes), wound induced gene promoters (e.g. hydroxyproline rich cell wall protein genes), chemically-inducible gene promoters (e.g., nitrate reductase genes, glucanase genes, chitinase genes, etc.) and dark-inducible gene promoters (e.g., asparagine synthetase gene) to name a few.

35 Tissue specific and development-specific promoters are also contemplated for use in the present invention. Examples of these included, but are not limited to: the ribulose bisphosphate carboxylase (RuBisCo) small subunit gene promoters or chlorophyll a/b binding protein (CAB) gene promoters for expression in photosynthetic tissue; the various seed storage protein gene promoters for expression in seeds; and the root-specific glutamine synthetase gene promoters where expression in roots is desired. Although the AAPK gene of the preferred embodiments taught with this invention are specifically expressed in guard cells, this in no way limits the

application of this invention to any specific tissue or development phase, but rather represents only the particular embodiments taught herein.

5 The coding region is also operably linked to an appropriate 3' regulatory sequence. In a preferred embodiment, the nopaline synthetase polyadenylation region (NOS) is used. Other useful 3' regulatory regions include, but are not limited to the octopine (OCS) polyadenylation region.

10 Using an *Agrobacterium* binary vector system for transformation, the selected coding region, under control of a constitutive or inducible promoter as described above, is linked to a nuclear drug resistance marker, such as kanamycin resistance. Other useful selectable marker systems include, but are not limited to: other genes that confer antibiotic resistances (e.g., resistance to hygromycin or bialaphos) or herbicide resistance (e.g., resistance to sulfonylurea, phosphinothricin, or glyphosate).

15 Plants are transformed and thereafter screened for one or more properties, including the lack of *AAPK* protein, *AAPK* mRNA, or altered stomatal aperture responses to ABA treatment. It should be recognized that the amount of expression, as well as the tissue-specific pattern of expression of the transgenes in transformed plants can vary depending on the position of their insertion into the nuclear genome. Such positional effects are well known in the art. For this reason, several nuclear transformants should be regenerated and tested for expression of the transgene.

20 Transgenic plants that exhibit one or more of the aforementioned desirable phenotypes can be used for plant breeding, or directly in agricultural or horticultural applications. Plants containing one transgene may also be crossed with plants containing a complementary transgene in order to produce plants with enhanced or combined phenotypes.

25 An alternative to the transgenic approach described above is the screening of populations of plant mutants of a variety of species, from which *AAPK* mutants can be isolated. Such populations can be made by chemical mutagenesis, radiation mutagenesis, and transposon or T-DNA insertion, as is well known in the art. In a preferred embodiment, the mutants would be null mutants having a phenotype comprising reduced or substantially absent stomatal closure in response to abscisic acid, but no reduction in stomatal closure response to darkness or CO₂. In yet another preferred embodiment, mutant are screened for the phenotypes related to overproduction of the *AAPK* gene product and/or increased sensitivity to ABA-induced stomatal closure.

30 The nucleic acids of the invention can be used to isolate or create *AAPK* mutants in a selected species. In species such as maize where transposon insertion lines are available, oligonucleotide primers can be designed to screen lines for insertions in the *AAPK* gene. Plants with transposon or T-DNA insertions in the *AAPK* gene are very likely to have lost the function of the gene product. Through

breeding, a plant line may then be developed that is homozygous for the non-functional copy or the altered copy of the *AAPK* gene. The PCR primers for this purpose are designed so that large portions of the coding sequence the *AAPK* gene are specifically amplified using the sequence of the *AAPK* gene from the species to be probed (see 5 Baumann et al., 1998, *Theor. Appl. Genet.* 97:729-734).

Other *AAPK*-like mutants can be isolated from mutant populations using the distinctive phenotype characterized in accordance with the present invention. This approach is particularly appropriate in plants with low ploidy numbers where the phenotype of a recessive mutation is more easily detected. In order to identify these 10 mutants, the population of plants would be exposed to abscisic acid (ABA) or analogs of the hormone. Plants would then be screened for phenotype of the *AAPK* mutants: the reduced stomatal closure in response to applied ABA, without a reduction in stomatal response to darkness or CO₂. That the phenotype is caused by an *AAPK* mutation is then established by molecular means well known in the art.

15 It will be appreciated that any of the aforementioned transformation or mutagenesis techniques may be applied to any selected plant species. Such species include, but are not limited to, agronomically important crop plants such as maize, wheat, rice, rye, oats, barley, soy and other beans, sorghum, sunflower, canola, tobacco and alfalfa; vegetable and fruit crop plants such as beet, carrot, cruciferous 20 vegetables, cucumber, grape, pea, potato, rutabaga, tomato, tomatillo and turnip; and horticulturally important plants such as aster, begonia, chrysanthemum, clover, lawn and turf grasses, mint and other herbs, and zinnia.

25 **B. Uses of *AAPK* nucleic acids, proteins, antibodies, *AAPK* mutants and transgenic plants**

30 **Nucleic acid molecules.** *AAPK* nucleic acids may be used for a variety of purposes in accordance with the present invention. DNA, RNA, or fragments thereof, may be used as probes to detect the presence and/or expression of *AAPK* genes. Methods in which *AAPK* nucleic acids may be utilized as probes for such assays include, but are not limited to: (1) *in situ* hybridization; (2) Southern hybridization (3) Northern hybridization; and (4) assorted amplification reactions such as polymerase chain reactions (PCR).

35 The *AAPK* nucleic acids of the invention may also be utilized as probes to identify related genes from other plant species. As is well known in the art, hybridization stringencies may be adjusted to allow hybridization of nucleic acid probes with complementary sequences of varying degrees of homology. As described above, *AAPK* nucleic acids may be used to advantage to produce large quantities of substantially pure *AAPK*, or selected portions thereof. The *AAPK* nucleic acids can 40 be used to identify and isolate other putative members of this novel ABA-mediated

stomatal aperture control signal cascade *in vivo*. A yeast two hybrid system can be used to identify proteins that physically interact with the AAPK protein, as well as isolate their nucleic acids. In this system, the sequence encoding the protein of interest is operably linked to the sequence encoding half of a activator protein. This construct 5 is used to transform a yeast cell library which has been transformed with DNA constructs that contain the coding sequence for the other half of the activator protein operably linked to a random coding sequence from the organism of interest. When the protein made by the random coding sequence from the library interacts with the protein of interest, the two halves of the activator protein are physically associated and form a 10 functional unit that activates the reporter gene. In accordance with the present invention, all or part of the AAPK coding sequence may be operably linked to the coding sequence of the first half of the activator, and the library of random coding sequences may be constructed with cDNA from *V. faba* and operably linked to the coding sequence of the second half of the activator protein. Several activator 15 protein/reporter genes are customarily used in the yeast two hybrid system. In a preferred embodiment, the bacterial repressor LexA DNA-binding domain and the Gal4 transcription activation domain fusion proteins associate to activate the *LacZ* reporter gene (see Clark et al., 1998, PNAS 95:5401-5406). Kits for the two hybrid system are also commercially available from Clontech, Palo Alto CA, among others.

20 In a preferred embodiment, interaction cloning is used identify proteins that physically interact with the AAPK protein and to isolate the nucleic acids encoding them. In this method, a cDNA expression library is screened for proteins that interact with the AAPK catalytic domain, or other selected domains that might be involved in protein-protein interactions. This is done using a filter binding assay and a labeled 25 peptide comprising the putative interacting site. Positive clones are then purified, amplified if necessary, and characterized.

Proteins and antibodies. The AAPK proteins of the present invention can be used to identify molecules with binding affinity for AAPK, which are likely to be novel participants in this resistance pathway. In these assays, the known 30 protein is allowed to form a physical interaction with the unknown binding molecule(s), often in a heterogenous solution of proteins. The known protein in complex with associated molecules is then isolated, and the nature of the associated protein(s) and/or other molecules is determined.

AAPK may also be generated as part of a fusion protein with one or 35 more other proteins, for example with a green fluorescent protein (GFP). Such fusion products may have utility from either or each part of the fusion molecule. For example, the easy detection of their presence is provided by the GFP moiety, while the specific kinase activity is retained by the AAPK moiety. Additionally they may allow convenient use of commercially available antibodies specific to the fused portion (e.g.

antiGFP antibodies are readily available.)

Antibodies that are immunologically specific for AAPK may be utilized in affinity chromatography to isolate the AAPK protein, to identify or quantify the AAPK protein utilizing techniques such as western blotting and ELISA, or to immunoprecipitate AAPK from a sample containing a mixture of proteins and other biological materials. The immuno-precipitation of AAPK is particularly advantageous when utilized to isolate affinity binding complexes of AAPK, as described above.

Mutants and Transgenic Plants. The *AAPK* mutants of the invention display altered sensitivity to ABA-induced stomatal closure, and therefore can be used to improve crop and horticultural plant species. The *AAPK* mutants taught in this invention are particularly valuable in that the mutation is very specific. The altered sensitivity is found only in guard cells, and stomatal closure by other means such as darkness or CO₂ is unaffected. Such mutants will therefore be particularly useful in crop and horticultural varieties in which reduction of moisture content is important. Examples of such crops include but are not limited to cereal grains such as corn, wheat, rye, oats, barley, and rice, soybeans and other beans, as well as other products such as hay and commercial seed. In most of these cases failure to adequately dry the crop due to weather or other conditions results in substantial losses. In other cases including but not limited to tobacco, dried fruits such as raisins and prunes, nuts, coffee, tea, cocoa, and many ornamental goods, the produce needs to be dried immediately after harvest prior and to use. In these cases again, the mutants of this invention may be of tremendous value to growers who could accelerate or control the rate of crop drying.

The *AAPK* mutants exhibit a decreased induction by ABA of normal stomatal closure. They therefore have influence over transpirational water loss in plants. It is therefore contemplated that in addition to the specific applications mentioned heretofore, these mutants will have myriad applications to other important plant problems especially in irrigated crops or other crops where water and yield are delicately balanced.

It is also trivial to one skilled in the art to extend this invention to the production of mutants with increased sensitivity to ABA-induced stomatal closure. These mutants are useful for a variety agronomic purposes. It is clear that such mutants would keep the stomatal aperture small, and would therefore experience reduced transpirational water loss. Such mutants can be used to help enhance tolerance to water stress or drought conditions. Such mutants could be the result of active site changes or modifications which allow them to respond to lower concentrations of ABA, or they could be the result of mutations in genes in a common regulatory pathway. Such mutants could also be the result of overexpression of the *AAPK* gene product via a transgenic modification such that expression of *AAPK* is driven by an

inducible promoter, a strong, highly active constitutive promoter, or by increasing the copy number of the gene in the plant. These approaches are all conceptually simple to one skilled in the art, and other approaches may be preferred for particular embodiments.

5 The *AAPK* mutants of the invention can be used to identify and isolate additional members of this ABA-regulation of transpiration pathway. Mutations that, when combined with *AAPK* mutations, suppress the mutant phenotype, are likely to interact directly with *AAPK*, or to compensate in some significant indirect way for the loss of *AAPK* function. Since *AAPK* is known to be a protein kinase with both
10 autophosphorylation and substrate phosphorylation, there are opportunities to identify other important members of the ABA signal cascade using the mutants of this present invention.

15 The transgenic plants of the invention are particularly useful in conferring the *AAPK* phenotype to many different plant species. In this manner, a host of plant species with enhanced disease resistance can be easily made, to be used as breeding lines or directly in commercial operations. Such plants can have uses as crop species, or for ornamental use.

20 A plant that has had functional *AAPK* transgenically depleted will exhibit the same altered sensitivity to ABA-induced stomatal closure as *AAPK* mutants. It is therefore contemplated that transgenic *AAPK*-phenotype plants will be used with in the same aforementioned manner as the *AAPK* mutants. A transgenic approach is advantageous because it allows *AAPK*-phenotype plants to be created quickly, without time-consuming mutant generation, selection, and back-crossing.

25 A plant that has had functional *AAPK* increased may have enhanced sensitivity to ABA-induced stomatal closure compared to wild-type plants. Plants with enhanced sensitivity to ABA-induced stomatal closure will be extremely valuable to agriculture and horticulture by allowing plants to better tolerate periods of restricted water or drought. Additionally, such mutants may provide the advantage of allowing produce to retain water as long as possible. For many fruits, vegetables and flowers,
30 including cut flowers, it would be advantageous to minimize water loss during the harvest, transport and distribution. Retail customers too would benefit from the extended shelf-life of such fruits, vegetables and flowers which would remain fresher for longer periods of time.

35 The following examples are provided to describe the invention in greater detail. They are intended to illustrate, not to limit, the invention.

EXAMPLE 1**Cloning and Characterization of ABA-Activated Protein Kinase-Encoding cDNA from Guard Cells**

5 This example describes the cloning and characterization of a *Vicia faba* complementary DNA, AAPK, encoding a guard cell-specific ABA-activated serine-threonine protein kinase (AAPK).

METHODS

10 **Isolation and Identification of ABA-Activated Protein Kinase (AAPK).** Guard cell protoplasts (2 X 10⁶; 99.6% pure) were prepared from *Vicia faba*. Protoplasts were treated with either darkness, ABA or elevated CO₂ concentrations prior to protein isolation. Protoplast proteins were extracted and subjected to 2-dimensional gel electrophoresis. Separation was via 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Kinase autophosphorylation activity of subsequently renatured proteins was detected by established methods..

15 The AAPK protein was excised from the 2-D gels (first dimension, nondenaturing PAGE) after digestion with trypsin. The AAPK peptides generated by trypsin digestion were subjected to peptide sequencing by tandem mass spectrometry 20 on a Finnigan LCQ quadrupole ion trap mass spectrometer.

25 **Cloning of ABA-Activated Protein Kinase (AAPK).** The gene encoding AAPK was cloned by screening a *V. faba* cDNA library with probes constructed based on peptide sequence data obtained from the mass spectrometry analysis.

30 Degenerate DNA primers whose design was based on sequences conserved between subdomain II of the protein kinase ABA1 (PKABA1) protein kinase subfamily and the AAPK sequence corresponding to protein kinase subdomain VIb, were synthesized. The degenerate primers {5'-TTGC(C/T)(A/G)T(G/C) AA(A/G)TACATCGAA-3' (SEQ ID NO:17) (forward primer, located in subdomain II), 5'-CCATC(C/T)A(A/G)NAGNGT(A/G)TTTTC-3' (SEQ ID NO:18)(reverse primer, located in subdomain VIb) where N= A+G+C+T} were used for reverse transcription-polymerase chain reaction (RT-PCR) using as template total RNA from guard cells. of *V. faba*. The PCR product was labelled with {³²P}dCTP.

35 This ³²P-labelled probe was then used to screen a *V. faba* guard cell cDNA library in λ-Zap II. A full length cDNA of the appropriate size to encode the AAPK was obtained. The cDNA was sequenced in both directions.

 Total RNA was isolated from purified guard cells, mesophyll cells, flowers, leaves and seeds of *V. faba*. Northern analysis was performed by standard

methods. The probe was the ^{32}P -labelled BglII-Csp45I fragment of the *AAPK* cDNA clone. This probe includes the sequence encoding the relatively unique AAPK amino terminal region and part of the 3' untranslated region of the cDNA.

5 **Functional Analysis of the AAPK gene.** Functional analysis of the *AAPK* gene product was complicated by the observation that ABA-activation of the AAPK does not occur *in vitro*. Prior treatment of intact guard cells with ABA was required to elicit active AAPK upon extraction. In light of this apparent requirement for an intact cellular signal system, DNA constructs were created to facilitate the analysis of expression and activity of *AAPK*.

10 **Creation of AAPK mutants and hybrid expression DNA constructs.** A construct encoding a green fluorescent protein (GFP)-tagged AAPK was made. This construct, pAAPK-GFP, was created by amplifying the AAPK coding sequence from the *AAPK* cDNA and inserting the amplified coding sequence downstream of the 35S promoter and upstream of, and in-frame with, the GFP coding sequence in the GFP expression vector, pGFP. The amplification was performed via 15 PCR with the primers 5'-GAATCTCCACTACGACGCCGTTACTTCCC-3' (SEQ ID NO:19) and 5'-CCGTGCAACCATGGATATGGCATATACAAT-3' (SEQ ID NO:20). NcoI was used for digestion and insertion.

20 Another DNA construct, pAAPK(K43A)-GFP was created. This construct contained a site-directed mutation of AAPK, such that the coding sequence for a highly conserved lysine residue (Lys⁴³), believed to be in the ATP-binding site of the kinase AAPK, was specifically modified to encode an alanine residue instead of the lysine. Such mutations have been shown to yield kinases with reduced or absent catalytic activity.

25 **Analysis of AAPK mutants and hybrid expression DNA constructs for ABA-activated protein kinase activity.** 1.5×10^7 *V. faba* guard cell protoplasts were transfected with either the vector, pGFP, or the constructs, pAAPK-GFP or pAAPK(K43A)-GFP by polyethylene glycol (PEG)-mediated DNA transfer. After uptake and expression, protoplasts were lysed and recombinant protein 30 was immunoprecipitated with anti-GFP peptide antibodies (Clontech) and protein A-Sepharose CL-4B (Amersham Pharmacia Biotech). Immunoprecipitated proteins were assayed for kinase activity using histone III-S (Sigma) as substrate.

35 **Analysis of AAPK mutants for stomatal aperture changes and anion channel activation.** *V. faba* leaves were biolistically transformed with the pGFP, pAAPK-GFP, or pAAPK(K43A)-GFP constructs. The *V. faba* leaves were bombarded with gold particles (Bio-Rad) coated with one of the DNA constructs. Bombardment was via a particle delivery system 1000/He (Bio-Rad) as described. (J.

Marc et al., 1998, *Plant Cell* 10:1927).

Abaxial epidermal peels were isolated and the transformed guard cells were assayed for ABA-mediated prevention of stomatal opening and for stomatal closure stimulated by ABA, CO₂ or darkness. Conditions were as in Assman, S., and Baskin, T. (1998) *J. Exp. Bot.* 49:163 except that the incubation solution was 10 mM MES, 30mM KCl, pH = 6.1, with or without 50 μ M $\{\pm\}$ *cis,trans*-ABA. For closure experiments, the transformed leaves were illuminated with 0.20 mmol m⁻² s⁻¹ white light for 2.5 h to open the stomata. The abaxial epidermal peels were placed in incubation solution and treated with either darkness, 25 μ M $\{\pm\}$ *cis,trans*-ABA or with 700 ppm CO₂ for 1 h.

Anion channel activation was measured in guard cell protoplasts. Whole-cell patch-clamp experiments were performed according to established methods. Pipette solution contained 100 mM KCl, 50mM tetramethylammonium, 2 mM MgCl, 6.7 mM EGTA-(Tris)₂, 3.35 mM CaCl₂, 10mM HEPES, pH=7.1 (Tris) and 5 mM Mg-ATP. Bath solution contained 40 mM CaCl₂, 2 mM MgCl and 10 mM MES-Tris pH5.6. Osmolalities were adjusted with sorbitol to 500 mosmol/kg (in the pipette) or 470 mosmol/kg (in the bath). Protein kinase inhibitor K-252a (Calbiochem) was prepared as a stock solution at 2 mM in dimethyl sulfoxide (DMSO).

20

RESULTS AND DISCUSSION

The *Vicia faba* ABA-Activated Protein Kinase (AAPK) is a 48kDa protein. AAPK was identified as a 48 kDa ABA-dependent and Ca²⁺-independent autophosphorylation spot with the in-gel kinase assay. The peptide fragment sequence information obtained is provided in Figure 1. Two sequenced AAPK peptides had similarity to the protein kinase ABA1 (PKABA1) protein kinase subfamily in subdomains I and VIb. PKABA1 is transcriptionally up-regulated by ABA.

30 Cloning of a guard-cell-specific AAPK gene encoding ABA-Activated Protein Kinase (AAPK). The RT-PCR of total guard cell RNA using the degenerate primers yielded a 310 base pair sequence which encoded the peptides sequences from the AAPK and also encoded a sequence similar to that of the PKABA1 subfamily from subdomains II to VIb. A full length cDNA of the appropriate size to encode the AAPK was obtained from the screening of the *V. faba* cDNA library with this probe.

35 The nucleotide sequence of the full length AAPK cDNA and the amino

acid sequence of the deduced AAPK protein are given in Figure 1. The deduced AAPK amino acid sequence shows the greatest homology to the PKABA1 subfamily. However, the predicted sequence also has unique regions.

5 Northern analysis showed that *AAPK* mRNA is expressed in guard cell protoplasts but not in mesophyll cell protoplasts, flowers, leaves, or seeds; true to the pattern of guard cell-specificity previously observed for AAPK activity. *AAPK* appears to be a single copy gene based on results from Southern analysis; however, further analysis may reveal the presence of more than one copy..

10 The *in situ* activation requirement for AAPK activity could be an indication that an intact cellular signal or cascade is required. Any discussion or explanation offered here is intended to provide clarity and is not intended to limit the invention in any way to one theory or avenue as to the mechanism or application.

15 **ABA-dependent autophosphorylation and ABA-activated substrate phosphorylation.** No histone phosphorylation was observed by the proteins immunoprecipitated from guard cells transformed with the control vector (pGFP) only. Phosphorylation activity of the fusion proteins AAPK-GFP and AAPK(K43A)-GFP was also determined. The immunoprecipitate from cells transfected with pAAPK-GFP showed histone phosphorylation activity which was significantly enhanced when the protoplasts were treated with ABA prior to isolation of 20 the proteins. The fusion product of pAAPK-GFP showed both ABA-dependent autophosphorylation and the ABA-activated histone phosphorylation, establishing that the cloned gene indeed encodes the observed biochemical activity of AAPK. The immunoprecipitate from cells transfected with pAAPK(K43A)-GFP, encoding the site-mutagenized AAPK(K43A), also showed ABA-dependent autophosphorylation 25 and ABA-activated histone phosphorylation activity, however the relative levels were significantly reduced as would be predicted from the sequence change.

30 **ABA-mediated stomatal aperture regulation and anion channel activation in AAPK mutants.** The transformed guard cells were identified by their green fluorescence. Transformation with pAAPK(K43A)-GFP eliminated ABA-induced stomatal closure, but had no effect on stomatal closure induced by CO₂ or darkness. Transformation with wild-type AAPK via the pAAPK-GFP vector had no measurable effect on either ABA-induced stomatal closure nor on ABA-inhibition of 35 stomatal opening.

35 In *V. faba* guard cells, ABA activated slow anion channels. Slow anion currents were identified by their characteristic time dependence, their reversal

potential and sensitivity to the anion channel blocker 5-nitro-2-(3-phenylpropylamino)benzoic acid. Not only was the typical decay in anion current reversed over time in the whole-cell configuration, but ABA also increased the anion current magnitude. In guard cells transformed with pGFP or pAAPK-GFP, anion currents were regulated normally (activated) by ABA. In guard cells transformed with pAAPK(K43A)-GFP, however, ABA-activation of anion channels was eliminated.

5 It is likely that the K43A mutant kinase competes with the activity of native AAPK in a dominant negative fashion. First, the kinase inhibitor K-252a inhibits (i) native AAPK activity, (ii) ABA-induced stomatal closure, and (iii) ABA regulation 10 of anion channels in untransformed cells, implying that the channels are indeed normally regulated by AAPK. Second, although dominant *abi1-1* and *abi2-1* mutations in ABI and ABI2 phosphatases confer ABA insensitivity to both anion channel activation and stomatal closure, recently identified recessive loss-of-function mutations in *ABI1* confer hypersensitivity to ABA. Thus, in wild-type plants an AAPK may mediate 15 ABA-induced anion channel activation and stomatal closure through a phosphorylation event, while *ABI1* opposes ABA action through a dephosphorylation event.

Neither wild-type nor mutant versions of recombinant AAPK affected ABA inhibition of stomatal opening (Table 1). ABA inhibition of stomatal opening and ABA promotion of stomatal closure may, therefore, employ different signaling 20 cascades. Alternatively, and in contrast to current theory, ABA activation of anion channels may not be required for ABA inhibition of stomatal opening.

Agronomically, loss of ABA-stimulated stomatal closure in plants transformed with mutant AAPK under control of an inducible promoter should allow accelerated and controlled desiccation of crops that are dried before harvest or 25 distribution. Basal levels of ABA remain even in irrigated crops; under these conditions, inhibition of AAPK activity should alleviate stomatal limitation of CO₂ uptake, and thus accelerate growth or increase yield.

Table 1. Overexpression of AAPK(K43A) in guard cells inhibits ABA-induced stomatal closure. *V. faba* leaves were transformed and stomatal responses measured. ABA treatment was 25 μ M (for closure) or 50 μ M (for opening) (\pm -c_{is}, *trans*-ABA, elevated CO₂, treatment was 700 ppm CO₂. Except for the darkness treatment, peels were illuminated (0.20 mmol m⁻²s⁻¹ white light) for the duration of each treatment. All numbers represent the change in half aperture of stomata as measured in micrometers. ND, not determined. Numbers in parentheses indicate sample sizes.

	GFP		AAPK-GFP		AAPK (K43A)-GFP	
	Transformed	Untransformed	Transformed	Untransformed	Transformed	Untransformed
ABA	-2.52 \pm 0.29 (36)	-2.54 \pm 0.35 (36)	-2.59 \pm 0.30 (36)	-2.58 \pm 0.24 (36)	-0.36 \pm 0.26 (56)*	-2.55 \pm 0.21 (56)
Control	0.10 \pm 0.09 (10)	0.09 \pm 0.09 (10)	0.11 \pm 0.10 (10)	0.12 \pm 0.10 (24)	0.12 \pm 0.11 (10)	0.13 \pm 0.10 (24)
CO ₂	ND	ND	ND	ND	-2.23 \pm 0.44 (36)	-2.31 \pm 0.46 (36)
Control	ND	ND	ND	ND	-0.09 \pm 0.09 (10)	-0.11 \pm 0.10 (10)
Darkness	ND	ND	ND	ND	-2.08 \pm 0.40 (36)	-2.08 \pm 0.46 (36)
Control	ND	ND	ND	ND	0.12 \pm 0.11 (10)	0.13 \pm 0.11 (10)
ABA	0.42 \pm 0.22 (36)	0.45 \pm 0.27 (36)	0.43 \pm 0.22 (36)	0.41 \pm 0.28 (36)	0.42 \pm 0.17 (46)	0.44 \pm 0.15 (46)

* Significantly different from untransformed cells treated with ABA ($P < 0.001$, Student's *t* test). Not significantly different from the AAPK(K43A)-GFP transformed ABA control ($P > 0.05$, Student's *t* test).

EXAMPLE 2
Identification of AAPK Genes
from *Arabidopsis thaliana*

5 **Screening.** Standard methods known to those skilled in the art for screening a genomic library were used. An *Arabidopsis* genomic library from CD4-8 *Landsberg erecta* from the *Arabidopsis* Biological Resource Center at Ohio State University was used.

10 The probe was the Nco I - Bgl II fragment (393 base pairs) of *V. faba* AAPK cDNA. The gel-purified Nco I - Bgl II fragment of AAPK cDNA was labeled by 32P-dCTP. This probe corresponds to sequences encoding the region from the aspartic acid residue (position 2, SEQ ID NO:2) to the arginine residue (position 132, SEQ ID NO:2) of the AAPK protein.

15 Nylon membranes containing the library were prehybridized with 5x SSC, 5x Denhardt's solution, 1% SDS, and 0.2% nonfat milk at 60 C for 2 hours and then hybridized in the same solution with the labeled probe at 60 C overnight. The membranes were washed with 2x SSC and 0.1% SDS twice for 15 minutes at 60 C, once in 2x SSC and 0.1% SDS, and once in 0.5X SSC and 0.1% SDS. The membranes were exposed to X-ray films and positive plaques were identified by 20 autoradiography.

 The positive clones were subcloned into pCR BlueScript vector and then analyzed by DNA sequencing and compared to known sequences to look for matches.

25 **Data Mining.** The BLAST program of the National Center for Biological Information (NCBI) was used with the blastx option to search the nr (nonredundant) databases of GenBank, EMBL and DDBJ for matches with AAPK sequence data. The parameters used for the search were: expect value, 10; matrix, BLOSUM62; filter, low complexity.

30 **Results.** Two independent genomic clones were identified from the screening of the *Arabidopsis* library. Sequencing and subsequent database searches established that these sequences originated from sequences embodied respectively in GenBank Accession Numbers L05562 and Protein Identification Accession Number CAA19877. The data mining with the AAPK cDNA sequence corroborated the results obtained through screening of the genomic library. In addition, the database 35 search revealed another, equally homologous, *Arabidopsis* nucleic acid sequence.

comprising the sequences embodied in GenBank Accession Number L05561. The L05561 sequences correspond to a region within the *Arabidopsis* BAC clone ALO31032 of chromosome 4.

Sequence comparisons revealed that the predicted polypeptide 5 encoded by L05562 has 75.4% identity with the amino acid sequence encoded by the *V. faba* AAPK cDNA, and the nucleotide sequence has 67.9% identity to the nucleotide sequence of the *V. faba* AAPK cDNA. The predicted polypeptide encoded by CAA19877 has 77.5% identity with the deduced *V. faba* AAPK amino acid sequence, and the nucleotide sequence is 68.7% identical to the nucleic acid 10 sequence of *V. faba* AAPK cDNA. Various alignments and additional information regarding sequence identity are set forth in Figures 2-4.

The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification without departure from 15 the scope of the appended claims.

We claim:

1. An isolated nucleic acid molecule encoding an ABA-activated protein kinase (AAPK), expressed predominantly in guard cells of a plant.
5
2. The nucleic acid molecule of claim 1, which is a cDNA.
3. The nucleic acid molecule of claim 1, which is genomic DNA comprising exons that form an open reading frame encoding the AAPK.
10
4. The nucleic acid molecule of claim 1, modified to encode a nonfunctional AAPK.
5. The nucleic acid molecule of claim 4, modified in a kinase domain
15 of the AAPK.
6. The nucleic acid molecule of claim 5, modified to remove a conserved lysine residue in subdomain II of the kinase domain.
7. The nucleic acid molecule of claim 2, which encodes a polypeptide having an amino acid sequence greater than 60% identical to a sequence selected from the group consisting of SEQ ID NOS: 2, 4, 5, 7 and 8.
20
8. The nucleic acid molecule of claim 7, which encodes a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NOS: 2, 4, 5, 7 and 8.
25
9. The nucleic acid molecule of claim 7, having a coding sequence greater than 60% identical to the coding sequence selected from the group consisting of SEQ ID NOS: 1, 3 and 6.
30
10. The nucleic acid molecule of claim 9, wherein the coding sequence is selected from the group consisting of SEQ ID NOS: 1, 3 and 6.

11. An oligonucleotide greater than 15 nucleotides in length, which hybridizes with either strand of the nucleic acid molecule of claim 1 under pre-determined hybridization conditions..

5 12. A polypeptide produced by expression of the nucleic acid molecule of claim 1.

10 13. Antibodies immunologically specific for the polypeptide of claim 12.

14. A vector for transforming a plant cell, comprising the nucleic acid molecule of claim 1.

15 15. A transformed plant cell comprising the vector of claim 14.

16. An isolated nucleic acid molecule having a sequence selected from the group consisting of:

- a) any of SEQ ID NOS:1, 3 and 6;
- b) a sequence that is at least 60% identical to any of SEQ ID NOS:1, 3 and 6;
- c) a sequence encoding a polypeptide having any of SEQ ID NOS:2, 4, 5, 7 and 8;
- d) a sequence encoding a polypeptide having a sequence at least 67% identical to any of SEQ ID NOS:2, 4, 5, 7 and 8; and
- e) a sequence that hybridizes with any of SEQ ID NOS:1, 3 and 6 under conditions comprising hybridization at 37-42°C in a solution comprising: 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide, and washing at 42-65° in 1X SSC and 1% SDS.

30 17. A polypeptide produced by the expression of the isolated nucleic acid molecule of claim 16.

35 18. Antibodies immunologically specific for the polypeptide of claim 17.

19. An oligonucleotide, having at least a 15 base pair nucleotide portion identical in sequence to a consecutive 15 base pair portion of the sequence of the isolated nucleic acid molecule of claim 16.

5 20. A vector for transforming a plant cell, comprising the nucleic acid molecule of claim 16.

21. A transformed plant cell comprising the vector of claim 20.

10 22. A genetically altered plant possessing decreased sensitivity to ABA-induced stomatal closure as compared with an equivalent but unaltered plant, comprising an AAPK that is largely nonfunctional or absent.

15 23. The genetically altered plant of claim 22, produced by subjecting a population of plants to mutagenesis and selecting a mutagenized plant wherein the AAPK is largely nonfunctional or absent.

20 24. The genetically altered plant of claim 22, produced by transforming cells of the plant with a transgene that causes the plant's endogenous AAPK to become largely nonfunctional or absent, and regenerating the plant from the transformed cells.

25 25. The genetically altered plant of claim 24, wherein expression of the transgene is inducible.

26. A genetically altered plant possessing increased sensitivity to ABA-induced stomatal closure as compared with an equivalent but unaltered plant, comprising an AAPK that is increased in amount or activity as compared with the unaltered plant.

30 27. The genetically altered plant of claim 26, produced by subjecting a population of plants to mutagenesis and selecting a mutagenized plant wherein the AAPK is largely nonfunctional or absent.

35 28. The genetically altered plant of claim 26, produced by transforming cells of the plant with a transgene that causes the plant's endogenous

AAPK to become largely nonfunctional or absent, and regenerating the plant from the transformed cells.

29. The genetically altered plant of claim 28, wherein expression of the
5 transgene is inducible.

30. A method to increase transpiration in a plant, comprising reducing or preventing function of an AAPK in guard cells of the plant, thereby reducing sensitivity of the plant to ABA-induced stomatal closure, resulting in the increased transpiration.

10

31. The method of claim 30, wherein the AAPK function is reduced or prevented by the addition of at least one transgene to the plant genome.

15 32. The method of claim 31, wherein the transgene is mutated and expression of the transgene produces a non-functional protein.

33. The method of claim 32, wherein the transgene is mutated in a kinase-encoding domain.

20 34. The method of claim 33, wherein the transgene encodes a sequence selected from the group consisting of SEQ ID NO:2, 4, 5, 7 and 8, modified to remove or change an active site lysine residue in kinase subdomain II.

25 35. The method of claim 31, wherein expression of the transgene produces part or all of an antisense strand of the nucleic acid molecule of claim 16.

36. The method of claim 31, wherein an additional transgene is added to the plant genome, which comprises a sense strand of the nucleic acid molecule of claim 16.

30

37. A fertile plant produced by the method of claim 30.

35 38. A method to decrease transpiration in a plant, comprising increasing amount or activity of an AAPK in guard cells of the plant, thereby increasing sensitivity of the plant to ABA-induced stomatal closure, resulting in the decreased transpiration.

39. The method of claim 38, wherein the AAPK amount or activity is increased by the addition of at least one transgene to the plant genome.

5

40. A fertile plant produced by the method of claim 38.

FIGURE 1



2/4
Figure 2

Query: 3 MPPPIMHSDSDRYDFVRDIGSGNFGVARLMTDKLTAVKYIERGDKIDENVKREIINH 62
M PIMHSDSDRY+ V+DIGSGNFGVARLM DK + +LVAVKYIERG+KIDENVKREIINH
Sbjct: 10 MDLPIMHSDSDRYELVKDIGSGNFGVARLMDKQSNELVAVKYIERGEKIDENVKREIINH 69

Query: 63 RSLRHPNIVRFKEVILPTPLAIVMEYASGGEMSADRISKAGRFTEDEARFFFQQLISGV 122
RSLRHPNIVRFKEVILPTPLAIVMEYASGG+ +RI AGRF+EDEARFFFQQLISGV
Sbjct: 70 RSLRHPNIVRFKEVILPTPLAIVMEYASGGELFERICNAGRFS EDEARFFFQQLISGV 129

Query: 123 YCHSMQVCHRDLKLENTLLDGDPALHLKICDFGYSKSSVLHSQPKSTVGT PAYIAPEVLL 182
YCH+MQVCHRDLKLENTLLDG PA LKICDFGYSKSSVLHSQPKSTVGT PAYIAPEVLL
Sbjct: 130 YCHAMQVCHRDLKLENTLLDGSPAPRLKICDFGYSKSSVLHSQPKSTVGT PAYIAPEVLL 189

Query: 183 KQEYDGKIA DVWSCGVTLYVMLVG SYPFEDPDNP KDFRKT IQRVLSVQY SVPDFVQISPE 242
K+EYDGK+ADVWSCGVTLYVMLVG+YPFEDP+ PK+FRKT I R+L+VQY++PD+V ISPE
Sbjct: 190 KKEYDGKVADVWSCGVTLYVMLVGAYPFEDPEEPKNFRKT IHRILNVQY AIPDYVHISPE 249

Query: 243 CRDIISRIFVFDPAERITIPEIMKNEWFRKNLPADLVNENIMDNQFEEPDQPMQSMDT IM 302
CR +ISRIFV DPA+RI+IPEI +EWF KNLPADL+N+N M QF+E DQP QS++ IM
Sbjct: 250 CRHLISRIFVADPAKRISIPEIRNHEWFLKNLPADLMNDNTMTTQFDESDQPGQSIEEIM 309

Query: 303 QIISEATVPAAGSYYFDEFIEVDEDMDEIDS DY-ELDV DSSGEIVYAI 349
QII+EATVP AG+ + ++ D+ + + + SD +LD+DSSGEIVYAI+
Sbjct: 310 QIIAEATVPPAGTQNLNHYLTDDDMEE DLES DLDL DIDSSGEIVYAM 357

3/4
Figure 3

Query: 1 MDMPPPIMHSDRYDFVRDIGSGNFGVARLMTDKLTAVKYIERGDKIDENVKREII 60
M + PIMHSDRYDFV+DIGSGNFGVARLMTD++TK+LVAVKYIERG+KIDENV+REII
Sbjct: 10 MPIDLPIMHSDRYDFVKDIGSGNFGVARLMTDRVTKELVAVKYIERGEKIDENVQREII 69

Query: 61 NHRSLRHPNIVRFKEVILTPTHLAIMEYASGGEMSDRISKAGRFTEDEARFFFQQLISG 120
NHRSLRHPNIVRFKEVILTP+HLAIMEYA+GGE+ +RI AGRF+EDEARFFFQQLISG
Sbjct: 70 NHRSLRHPNIVRFKEVILTPSHLAIMEYAAGGELYERICNAGRFS EDEARFFFQQLISG 129

Query: 121 VSYCHSMQVCHRDLKLENTLLDGDPAHLKICDFGYSKSSVLHSQPKSTVGT PAYIAPEV 180
VSYCH+MQ+CHRDLKLENTLLDG PA LKICDFGYSKSSVLHSQPKSTVGT PAYIAPE+
Sbjct: 130 VSYCHAMQICHRDLKLENTLLDGSPAPRLKICDFGYSKSSVLHSQPKSTVGT PAYIAPEI 189

Query: 181 LLKQEYDGKIA DVWSCGVTLYVMLVGSYPFEDPDNPKDFRKTIQRVLSVQYSVPDFVQIS 240
LL+QEYDGK+ADVWSCGVTLYVMLVG+YPFEDP P+D+RKTIQR+LSV YS+P+ +S
Sbjct: 190 LLRQEYDGKLADWSCGVTLYVMLVGAYPFEDPQEPRDYRKTIQRILSVTYSIPEDLHLS 249

Query: 241 PECRDIISRIFVFDPAERITIPEIMKNEWFRKNLPADLVNENIMDNQFEEPDQPMQSMDT 300
PECR +ISRIIFV DPA RITIPEI ++WF KNLP DL++EN M +QF+EP+QPMQS+DT
Sbjct: 250 PECRHLISRIFVADPATRITIPEITSDFKWLKNLPGDLMDENRMGSQFQEPEQPMQSLDT 309

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Sbjct: 310 IMQIISEATIPTVRNCLDDFMADNLDDDDFDSESEIDVDSSGEIVYAL 362

4/4
Figure 4

Query: 1 MDMPPPIMHSDSDRYDFVRDIGSGNFGVARLMTDKLTDLVAVKYIERGDKIDENVKREII 60
M + PIMHSDSDRYDFV+DIGSGNFGVARLMTD++TK+LVAVKYIERG+KIDENV+REII
Sbjct: 10 MPIDLPIMHSDSDRYDFVKDIGSGNFGVARLMTDRVTKELVAVKYIERGEKIDENVQREII 69

Query: 61 NHRSLRHPNIVRFKEVILTPTHLAIIVMEYASGGEMSDRISKAGRFTEDEARFFFQQLISG 120
NHRSLRHPNIVRFKEVILTP+HLAIIVMEYA+GGE+ +RI AGRF+EDEARFFFQQLISG
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Sbjct: 190 LLRQEYDGKLADVWSCGVTLYVMLVGAYPFEDPQEPRDYRKTIQRILSVTYSIPEDLHLS 249

Query: 241 PECDIISRIFVFDPAERITIPEIMKNEWFRKNLPADLVNENIMDNQFEEPQPMQSMDT 300
PECR +ISRIFV DPA RITIPEI ++WF KNLP DL++EN M +QF+EP+QPMQS+DT
Sbjct: 250 PECRHLISRIFVADPATRITIPEITSDFWFLKNLPGDLMDENRMGSQFQEPEQPMQSLDT 309

Query: 301 IMQIISEATVPAAGSYYFDEF---IEVDEDMDIEIDSDYELDVDSSEIVYAI 349
IMQIISEAT+P + D+F +++D+DMD+ DS+ E+DVDSSGEIVYA+
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SEQUENCE LISTING

<110> Assmann, Sarah M.
Li, Jiaxu

<120> Compositions and Methods for Regulating
Abscisic Acid-Induced Closure of Plant Stomata

<130> PSU 99-2140

<150> US 60/142,039
<151> 1999-07-01

<150> US 60/176,245
<151> 2000-01-14

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 Asp Lys Ile Asp Glu Asn Val Lys Arg Glu Ile Ile Asn His Arg Ser
 50 55 60
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 Val Cys His Arg Asp Leu Lys Leu Glu Asn Thr Leu Leu Asp Gly Asp
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 145 150 155 160
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 Ala Pro Glu Val Leu Leu Lys Gln Glu Tyr Asp Gly Lys Ile Ala Asp
 180 185 190
 Val Trp Ser Cys Gly Val Thr Leu Tyr Val Met Leu Val Gly Ser Tyr
 195 200 205
 Pro Phe Glu Asp Pro Asp Asn Pro Lys Asp Phe Arg Lys Thr Ile Gln
 210 215 220
 Arg Val Leu Ser Val Gln Tyr Ser Val Pro Asp Phe Val Gln Ile Ser
 225 230 235 240
 Pro Glu Cys Arg Asp Ile Ile Ser Arg Ile Phe Val Phe Asp Pro Ala
 245 250 255
 Glu Arg Ile Thr Ile Pro Glu Ile Met Lys Asn Glu Trp Phe Arg Lys
 260 265 270
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 Glu Glu Pro Asp Gln Pro Met Gln Ser Met Asp Thr Ile Met Gln Ile
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 Ile Ser Glu Ala Thr Val Pro Ala Ala Gly Ser Tyr Tyr Phe Asp Glu
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tcttatgaga gataaaactca ctaaagagct tgggtgtgc aagtacatcg agagaggaga	300
caagattgtat gaaaatgttc aaagggagat cattaaccac aggtcaactaa ggcacatc	360
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<211> 361

<212> PRT

<213> *Arabidopsis thaliana*

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Phe Lys Glu Val Ile Leu Thr Pro Thr His Leu Ala Ile Ile Met Glu	
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Glu Asn Thr Leu Leu Asp Gly Ser Pro Ala Pro Arg Leu Lys Ile Cys	
145 150 155 160	
Asp Phe Gly Tyr Ser Lys Ser Ser Val Leu His Ser Gln Pro Lys Ser	
165 170 175	
Thr Val Gly Thr Pro Ala Tyr Ile Ala Pro Glu Val Leu Leu Arg Gln	
180 185 190	
Glu Tyr Asp Gly Lys Ile Ala Asp Val Trp Ser Cys Gly Val Thr Leu	
195 200 205	
Tyr Val Met Leu Val Gly Ala Tyr Pro Phe Glu Asp Pro Glu Glu Pro	

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225	230	235	
Ile Pro Asp Asp Ile Arg Ile Ser Pro Glu Cys Cys His Leu Ile Ser		240	
245	250	255	
Arg Ile Phe Val Ala Asp Pro Ala Thr Arg Ile Ser Ile Pro Glu Ile			
260	265	270	
Lys Thr His Ser Trp Phe Leu Lys Asn Leu Pro Ala Asp Leu Met Asn			
275	280	285	
Glu Ser Asn Thr Gly Ser Gln Phe Gln Glu Pro Glu Gln Pro Met Gln			
290	295	300	
Ser Leu Asp Thr Ile Met Gln Ile Ile Ser Glu Ala Thr Ile Pro Ala			
305	310	315	320
Val Arg Asn Arg Cys Leu Asp Asp Phe Met Thr Asp Asn Leu Asp Leu			
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Gly Val Ala Arg Leu Met Arg Asp Lys Gln Ser Asn Glu Leu Val Ala			
35	40	45	
Val Lys Tyr Ile Glu Arg Gly Glu Lys Ile Asp Glu Asn Val Lys Arg			
50	55	60	
Glu Ile Ile Asn His Arg Ser Leu Arg His Pro Asn Ile Val Arg Phe			
65	70	75	80
Lys Glu Val Ile Leu Thr Pro Thr His Leu Ala Ile Val Met Glu Tyr			
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Ala Ser Gly Gly Glu Leu Phe Glu Arg Ile Cys Asn Ala Gly Arg Phe			
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Ser Glu Asp Glu Ala Arg Phe Phe Gln Gln Leu Ile Ser Gly Val			
115	120	125	
Ser Tyr Cys His Ala Met Gln Val Cys His Arg Asp Leu Lys Leu Glu			
130	135	140	
Asn Thr Leu Leu Asp Gly Ser Pro Ala Pro Arg Leu Lys Ile Cys Asp			
145	150	155	160
Phe Gly Tyr Ser Lys Ser Ser Val Leu His Ser Gln Pro Lys Ser Thr			
165	170	175	
Val Gly Thr Pro Ala Tyr Ile Ala Pro Glu Val Leu Leu Lys Lys Glu			
180	185	190	
Tyr Asp Gly Lys Val Ala Asp Val Trp Ser Cys Gly Val Thr Leu Tyr			
195	200	205	
Val Met Leu Val Gly Ala Tyr Pro Phe Glu Asp Pro Glu Glu Pro Lys			
210	215	220	
Asn Phe Arg Lys Thr Ile His Arg Ile Leu Asn Val Gln Tyr Ala Ile			
225	230	235	240
Pro Asp Tyr Val His Ile Ser Pro Glu Cys Arg His Leu Ile Ser Arg			
245	250	255	

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 260 265 270
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 275 280 285
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 290 295 300
 Ile Glu Glu Ile Met Gln Ile Ile Ala Glu Ala Thr Val Pro Pro Ala
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 Gly Thr Gln Asn Leu Asn His Tyr Leu Thr Asp Asp Asp Met Glu Glu
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 Asp Leu Glu Ser Asp Leu Asp Asp Leu Asp Ile Asp Ser Ser Gly Glu
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 Asn Phe Gly Val Ala Arg Leu Met Thr Asp Arg Val Thr Lys Glu Leu

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Gln Arg Glu Ile Ile Asn His Arg Ser Leu Arg His Pro Asn Ile Val		
65	70	75
Arg Phe Lys Glu Val Ile Leu Thr Pro Ser His Leu Ala Ile Val Met		80
85	90	95
Glu Tyr Ala Ala Gly Gly Glu Leu Tyr Glu Arg Ile Cys Asn Ala Gly		
100	105	110
Arg Phe Ser Glu Asp Glu Ala Arg Phe Phe Gln Gln Leu Ile Ser		
115	120	125
Gly Val Ser Tyr Cys His Ala Met Gln Ile Cys His Arg Asp Leu Lys		
130	135	140
Leu Glu Asn Thr Leu Leu Asp Gly Ser Pro Ala Pro Arg Leu Lys Ile		
145	150	155
Cys Asp Phe Gly Tyr Ser Lys Ser Ser Val Leu His Ser Gln Pro Lys		
165	170	175
Ser Thr Val Gly Thr Pro Ala Tyr Ile Ala Pro Glu Ile Leu Leu Arg		
180	185	190
Gln Glu Tyr Asp Gly Lys Leu Ala Asp Val Trp Ser Cys Gly Val Thr		
195	200	205
Leu Tyr Val Met Leu Val Gly Ala Tyr Pro Phe Glu Asp Pro Gln Glu		
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Pro Arg Asp Tyr Arg Lys Thr Ile Gln Arg Ile Leu Ser Val Thr Tyr		
225	230	235
Ser Ile Pro Glu Asp Leu His Leu Ser Pro Glu Cys Arg His Leu Ile		
245	250	255
Ser Arg Ile Phe Val Ala Asp Pro Ala Thr Arg Ile Thr Ile Pro Glu		
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Ile Thr Ser Asp Lys Trp Phe Leu Lys Asn Leu Pro Gly Asp Leu Met		
275	280	285
Asp Glu Asn Arg Met Gly Ser Gln Phe Gln Glu Pro Glu Gln Pro Met		
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Gln Ser Leu Asp Thr Ile Met Gln Ile Ile Ser Glu Ala Thr Ile Pro		
305	310	315
Thr Val Arg Asn Arg Cys Leu Asp Asp Phe Met Ala Asp Asn Leu Asp		
325	330	335
Leu Asp Asp Asp Met Asp Asp Phe Asp Ser Glu Ser Glu Ile Asp Val		
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<211> 362

<212> PRT

<213> *Arabidopsis thaliana*

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Val Ala Val Lys Tyr Ile Glu Arg Gly Glu Lys Ile Asp Glu Asn Val		
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Gln Arg Glu Ile Ile Asn His Arg Ser Leu Arg His Pro Asn Ile Val		
65	70	75
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Arg Phe Lys Glu Val Ile Leu Thr Pro Ser His Leu Ala Ile Val Met
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 Glu Tyr Ala Ala Gly Gly Glu Leu Tyr Glu Arg Ile Cys Asn Ala Gly
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 Arg Phe Ser Glu Asp Glu Ala Arg Phe Phe Gln Gln Leu Ile Ser
 115 120 125
 Gly Val Ser Tyr Cys His Ala Met Gln Ile Cys His Arg Asp Leu Lys
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 Cys Asp Phe Gly Tyr Ser Lys Ser Ser Val Leu His Ser Gln Pro Lys
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 Ser Thr Val Gly Thr Pro Ala Tyr Ile Ala Pro Glu Ile Leu Leu Arg
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 Gln Glu Tyr Asp Gly Lys Leu Ala Asp Val Trp Ser Cys Gly Val Thr
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gttctgggtc acaaattctc tgtagcctac	agattggctt gatgtgtaaa	cagtgtaa	1140
taagtttaca tgcttatatac gaaatcagta	gttttacccg	aaaaaaaaaa	1200
aaaaaaaaaa			1210

<210> 10
 <211> 332
 <212> PRT
 <213> *Triticum aestivum*

<400> 10															
Gly	Ser	Gly	Asn	Phe	Gly	Val	Ala	Lys	Leu	Val	Arg	Asp	Val	Arg	Thr
1															15
Lys	Glu	His	Phe	Ala	Val	Lys	Phe	Ile	Glu	Arg	Gly	His	Lys	Ile	Asp
															20
															25
															30
Glu	His	Val	Gln	Arg	Glu	Ile	Met	Asn	His	Arg	Ser	Leu	Lys	His	Pro
															35
															40
															45
Asn	Ile	Ile	Arg	Phe	Lys	Glu	Val	Val	Leu	Thr	Pro	Thr	His	Leu	Ala
															50
															55
Ile	Val	Met	Glu	Tyr	Ala	Ser	Gly	Gly	Glu	Leu	Phe	Gln	Arg	Ile	Cys
															65
															70
															75
															80
Asn	Ala	Gly	Arg	Phe	Ser	Glu	Asp	Glu	Gly	Arg	Phe	Phe	Phe	Gln	Gln
															85
															90
															95
Leu	Ile	Ser	Gly	Val	Ser	Tyr	Cys	His	Ser	Met	Gln	Val	Cys	His	Arg
															100
															105
															110
Asp	Leu	Lys	Leu	Glu	Asn	Thr	Leu	Leu	Asp	Gly	Ser	Val	Ala	Pro	Arg
															115
															120
															125
Leu	Lys	Ile	Cys	Asp	Phe	Gly	Tyr	Ser	Lys	Ser	Ser	Val	Leu	His	Ser
															130
															135
															140
Gln	Pro	Lys	Ser	Thr	Val	Gly	Thr	Pro	Ala	Tyr	Ile	Ala	Pro	Glu	Val
															145
															150
															155
Leu	Ser	Arg	Arg	Glu	Tyr	Asp	Gly	Lys	Val	Ala	Asp	Val	Trp	Ser	Cys
															165
															170
															175
Gly	Val	Thr	Leu	Tyr	Val	Met	Leu	Val	Gly	Ala	Tyr	Pro	Phe	Glu	Asp
															180
															185
															190
Pro	Asp	Glu	Pro	Arg	Asn	Phe	Arg	Lys	Thr	Ile	Thr	Arg	Ile	Leu	Ser
															195
															200
															205
Val	Gln	Tyr	Ser	Val	Pro	Asp	Tyr	Val	Arg	Val	Ser	Met	Asp	Cys	Ile
															210
															215
															220
His	Leu	Leu	Ser	Arg	Ile	Phe	Val	Gly	Asn	Pro	Gln	Gln	Arg	Ile	Thr
															225
															230
															235
Ile	Pro	Glu	Ile	Lys	Asn	His	Pro	Trp	Phe	Leu	Lys	Arg	Leu	Pro	Val
															245
															250
															255
Glu	Met	Thr	Asp	Glu	Tyr	Gln	Arg	Ser	Met	Gln	Leu	Ala	Asp	Met	Asn
															260
															265
															270
Thr	Pro	Ser	Gln	Ser	Leu	Glu	Glu	Ala	Met	Ala	Ile	Ile	Gln	Glu	Ala
															275
															280
															285
Gln	Lys	Pro	Gly	Asp	Asn	Ala	Leu	Gly	Val	Ala	Gly	Gln	Val	Ala	Cys
															290
															295
Leu	Gly	Ser	Met	Asp	Leu	Asp	Asp	Ile	Asp	Phe	Asp	Ile	Asp	Asp	Ile
															305
															310
															315
Asp	Val	Glu	Ser	Ser	Gly	Asp	Phe	Val	Cys	Pro	Leu				
															325
															330

<210> 11
 <211> 339

<212> PRT

<213> Nicotiana tabacum

<400> 11

Met Glu Glu Lys Tyr Glu Leu Leu Lys Glu Leu Gly Thr Gly Asn Phe
 1 5 10 15
 Gly Val Ala Arg Leu Val Lys Asp Lys Lys Thr Lys Glu Leu Phe Ala
 20 25 30
 Val Lys Tyr Ile Glu Arg Gly Lys Lys Ile Asp Glu Asn Val Gln Arg
 35 40 45
 Glu Ile Ile Asn His Arg Ser Leu Gly His Pro Asn Ile Ile Arg Phe
 50 55 60
 Lys Glu Val Leu Val Thr Pro Ser His Leu Ala Ile Val Met Glu Tyr
 65 70 75 80
 Ala Ala Gly Gly Glu Leu Phe Ala Arg Ile Cys Ser Ala Gly Arg Phe
 85 90 95
 Ser Glu Asp Glu Ala Arg Phe Phe Gln Gln Leu Ile Ser Gly Val
 100 105 110
 Ser Tyr Cys His Ala Met Glu Ile Cys His Arg Asp Leu Lys Leu Glu
 115 120 125
 Asn Thr Leu Leu Asp Gly Ser Ala Ser Pro Arg Val Lys Ile Cys Asp
 130 135 140
 Phe Gly Tyr Ser Lys Ser Gly Leu Leu His Ser Gln Pro Lys Ser Thr
 145 150 155 160
 Val Gly Thr Pro Ala Tyr Ile Ala Pro Glu Val Leu Ser Arg Lys Glu
 165 170 175
 Tyr Asp Gly Lys Ile Ala Asp Val Trp Ser Cys Gly Val Thr Leu Tyr
 180 185 190
 Val Met Leu Val Gly Ala Tyr Pro Phe Glu Asp Pro Glu Asp Pro Lys
 195 200 205
 Asn Phe Arg Lys Thr Ile Gly Arg Ile Met Ser Ala Gln Tyr Ser Ile
 210 215 220
 Pro Asp Tyr Val Arg Ile Ser Ala Asp Cys Lys Asn Leu Leu Ser Arg
 225 230 235 240
 Ile Phe Val Ala Asn Pro Ser Lys Arg Ile Thr Ile Pro Glu Ile Lys
 245 250 255
 Lys His Pro Trp Phe Leu Lys Asn Leu Pro Lys Asp Leu Met Asp Gly
 260 265 270
 Glu His Ser Lys Tyr Glu Glu Ala Ser Glu Gln Leu Gln Ser Val
 275 280 285
 Glu Glu Ile Met Arg Ile Ile Gln Glu Ala Lys Ile Pro Gly Glu Val
 290 295 300
 Ser Lys Pro Glu Gly Gln Ala Thr Ala Gly Thr Ala Glu Pro Asp Asp
 305 310 315 320
 Thr Glu Asp Asp Leu Glu Ser Glu Ile Asp Ser Ser Asn Asp Phe Ala
 325 330 335
 Val Tyr Val

<210> 12

<211> 349

<212> PRT

<213> Glycine max

<400> 12

Met Asp Lys Tyr Glu Ala Val Lys Asp Leu Gly Ala Gly Asn Phe Gly
 1 5 10 15
 Val Ala Arg Leu Met Arg Asn Lys Val Thr Lys Glu Leu Val Ala Met

20	25	30
Lys Tyr Ile Glu Arg Gly Pro Lys Ile Asp Glu Asn Val Ala Arg Glu		
35	40	45
Ile Met Asn His Arg Ser Leu Arg His Pro Asn Ile Ile Arg Tyr Lys		
50	55	60
Glu Val Val Leu Thr Pro Thr His Leu Ala Ile Val Met Glu Tyr Ala		
65	70	75
Ala Gly Gly Glu Leu Phe Glu Arg Ile Cys Ser Ala Gly Arg Phe Ser		
85	90	95
Glu Asp Glu Ala Arg Tyr Phe Phe Gln Gln Leu Ile Ser Gly Val His		
100	105	110
Phe Cys His Thr Met Gln Ile Cys His Arg Asp Leu Lys Leu Glu Asn		
115	120	125
Thr Leu Leu Asp Gly Ser Pro Ala Pro Arg Leu Lys Ile Cys Asp Phe		
130	135	140
Gly Tyr Ser Lys Ser Ser Leu Leu His Ser Arg Pro Lys Ser Thr Val		
145	150	155
Gly Thr Pro Ala Tyr Ile Ala Pro Glu Val Leu Ser Arg Arg Glu Tyr		
165	170	175
Asp Gly Lys Leu Ala Asp Val Trp Ser Cys Ala Val Thr Leu Tyr Val		
180	185	190
Met Leu Val Gly Ala Tyr Pro Phe Glu Asp Gln Asp Asp Pro Arg Asn		
195	200	205
Phe Arg Lys Thr Ile Gln Arg Ile Met Ala Val Gln Tyr Lys Ile Pro		
210	215	220
Asp Tyr Val His Ile Ser Gln Asp Cys Arg His Leu Leu Ser Arg Ile		
225	230	235
Phe Val Ala Asn Pro Leu Arg Arg Ile Thr Ile Lys Glu Ile Lys Asn		
245	250	255
His Pro Trp Phe Leu Arg Asn Leu Pro Arg Glu Leu Thr Glu Ser Ala		
260	265	270
Gln Ala Ile Tyr Tyr Gln Arg Asp Ser Pro Asn Phe His Leu Gln Ser		
275	280	285
Val Asp Glu Ile Met Lys Ile Val Gly Glu Ala Arg Asn Pro Pro Pro		
290	295	300
Val Ser Arg Ala Leu Lys Gly Phe Gly Trp Glu Gly Glu Glu Asp Leu		
305	310	315
Asp Glu Glu Val Glu Glu Glu Asp Glu Asp Glu Tyr Asp Lys Arg		
325	330	335
Val Lys Glu Val His Ala Ser Gly Glu Phe Gln Ile Ser		
340	345	

<210> 13
 <211> 334
 <212> PRT
 <213> Oryza sativa

<400> 13		
Met Glu Glu Arg Tyr Glu Ala Leu Lys Glu Leu Gly Ala Gly Asn Phe		
1	5	10
Gly Val Ala Arg Leu Val Arg Asp Lys Arg Ser Lys Glu Leu Val Ala		
20	25	30
Val Lys Tyr Ile Glu Arg Gly Lys Lys Ile Asp Glu Asn Val Gln Arg		
35	40	45
Glu Ile Ile Asn His Arg Ser Leu Arg His Pro Asn Ile Ile Arg Phe		
50	55	60
Lys Glu Val Cys Leu Thr Pro Thr His Leu Ala Ile Val Met Glu Tyr		
65	70	75
		80

Ala Ala Gly Gly Glu Leu Phe Glu Gln Ile Cys Thr Ala Gly Arg Phe
 85 90 95
 Ser Glu Asp Asp Ala Arg Tyr Phe Phe Gln Gln Leu Ile Ser Gly Val
 100 105 110
 Ser Tyr Cys His Ser Leu Glu Ile Cys His Arg Asp Leu Lys Leu Glu
 115 120 125
 Asn Thr Leu Leu Asp Gly Ser Pro Thr Pro Arg Val Lys Ile Cys Asp
 130 135 140
 Phe Gly Tyr Ser Lys Ser Ala Leu Leu His Ser Lys Pro Lys Ser Thr
 145 150 155 160
 Val Gly Thr Pro Ala Tyr Ile Ala Pro Glu Val Leu Ser Arg Lys Glu
 165 170 175
 Tyr Asp Gly Lys Val Ala Asp Val Trp Ser Cys Gly Val Thr Leu Tyr
 180 185 190
 Val Met Leu Val Gly Ser Tyr Pro Phe Glu Asp Pro Gly Asp Pro Arg
 195 200 205
 Asn Phe Arg Lys Thr Ile Ser Arg Ile Leu Gly Val Gln Tyr Ser Ile
 210 215 220
 Pro Asp Tyr Val Arg Val Ser Ser Asp Cys Arg Arg Leu Leu Ser Gln
 225 230 235 240
 Ile Phe Val Ala Asp Pro Ser Lys Arg Ile Thr Ile Pro Glu Ile Lys
 245 250 255
 Lys His Thr Trp Phe Leu Lys Asn Leu Pro Lys Glu Ile Ser Glu Arg
 260 265 270
 Glu Lys Ala Asp Tyr Lys Asp Thr Asp Ala Ala Pro Pro Thr Gln Ala
 275 280 285
 Val Glu Glu Ile Met Arg Ile Ile Gln Glu Gly Lys Val Pro Gly Asp
 290 295 300
 Met Ala Ala Ala Asp Pro Ala Leu Leu Ala Glu Leu Ala Glu Leu Lys
 305 310 315 320
 Ser Asp Asp Glu Glu Ala Ala Asp Glu Tyr Asp Thr Tyr
 325 330

<210> 14
 <211> 342
 <212> PRT
 <213> *Mesembryanthemum crystallinum*

<400> 14

Met Glu Leu Tyr Glu Ile Val Lys Asp Ile Gly Ser Gly Asn Phe Gly
 1 5 10 15
 Gln Ala Lys Leu Val Arg Asp Lys Trp Thr Asn Glu Phe Val Ala Val
 20 25 30
 Lys Phe Ile Glu Arg Gly Ser Lys Asp Asp Glu His Val Gln Arg Lys
 35 40 45
 Leu Met Asn His Ser Ser Leu Lys His Pro Asn Ile Ile Arg Phe Lys
 50 55 60
 Glu Val Leu Leu Thr Pro Thr His Leu Ala Ile Val Met Glu Tyr Ala
 65 70 75 80
 Ala Gly Gly Glu Leu Phe Glu Arg Ile Cys Asn Ala Gly Arg Phe Arg
 85 90 95
 Glu Asp Glu Ala Arg Phe Phe Gln Gln Leu Ile Ser Gly Val Ser
 100 105 110
 Tyr Cys His Ser Met Gln Ile Cys His Arg Asp Leu Lys Leu Glu Asn
 115 120 125
 Thr Leu Leu Asp Gly Ser Pro Ala Pro Arg Val Lys Ile Cys Asp Phe
 130 135 140
 Gly Tyr Ser Lys Ser Ser Val Leu His Ser Gln Pro Lys Ser Ala Val

145	150	155	160
Gly Thr Pro Ala Tyr Ile Ala Pro Glu Val Leu Ser Lys Arg Glu Tyr			
165	170	175	
Asp Gly Lys Ile Ala Asp Val Trp Ser Cys Gly Val Thr Leu Tyr Val			
180	185	190	
Met Leu Phe Gly Ala Tyr Pro Phe Glu Asp Pro Asp Asp Pro Lys Asn			
195	200	205	
Phe Arg Lys Ser Leu Val Arg Ile Leu Ser Val Gln Tyr Cys Ile Pro			
210	215	220	
Asp Asn Ile Pro Ile Ser Met Glu Cys Arg His Leu Leu Ser Arg Ile			
225	230	235	240
Phe Val Ala Asn Pro Glu Lys Arg Ile Thr Ile Pro Glu Ile Lys Asn			
245	250	255	
His Pro Trp Phe Gln Lys Asn Leu Pro Met Glu Leu Met Glu Gly Gly			
260	265	270	
Ser Trp Gln Ser His Asp Ile Asn His Pro Ser Gln Asn Ile Gly Glu			
275	280	285	
Ile Leu Ser Ile Ile Gln Glu Ala Arg Gln Pro Ala Glu Leu Pro Ser			
290	295	300	
Thr Gly Gly Leu Gln Ile Gly Gly Thr Leu Asp Phe Asp Asp Leu Asp			
305	310	315	320
Val Asp Leu Asp Val Asp Val Asp Leu Asp Asp Ile Glu Ser Ser Gly			
325	330	335	
Glu Phe Val Cys Pro Met			
340			

<210> 15

<211> 11

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic Sequence

<400> 15

Pro Ile Met His Asp Ser Asp Arg Tyr Asp Phe		
1	5	10

<210> 16

<211> 19

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic Sequence

<400> 16

Pro Ala Asp Leu Val Asn Glu Asn Ile Met Asp Asn Gln Phe Glu Glu		
1	5	10
Pro Asp Gln		15

<210> 17

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic Sequence

<400> 17
ttgcyrtyaa rtacatcgaa 20

<210> 18
<211> 20
<212> DNA
<213> Artificial Sequence

<400> 18
ccatcyarna gngtrttttc 20

<210> 19
<211> 30
<212> DNA
<213> Artificial Sequence

<400> 19
gaatctccac tacgacgccc tttacttccc 30

<210> 20
<211> 30
<212> DNA
<213> Artificial Sequence

<400> 20
ccgtgcaacc atggatatgg catatacaat 30